ECOLOGICAL AND MOLECULAR CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES OBTAINED FROM WATERFOWL ON THE TEXAS COAST

A Dissertation

by

PAMELA JOYCE BLOOMER FERRO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Veterinary Microbiology

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Approved by:

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	Markus J. Peterson
Committee Members,	John El-Attrache
	Thomas L. Lester
Head of Department,	John August

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ABSTRACT

Ecological and Molecular Characterization of Avian Influenza Viruses Obtained from Waterfowl on the Texas Coast.

(August 2010)

Pamela Joyce Bloomer Ferro

B.S.; M.S., Texas A&M University;

Co-Chairs of Committee: Dr. Blanca Lupiani
Dr. Markus J. Peterson

We collected 6,823 cloacal swabs over four years (2005–2006: 1,460; 2006–2007: 2,171; 2007–2008: 2,424; and 2008–2009: 768) from 30 potential avian host species. Most samples (88.3%) were from dabbling ducks (genus *Anas*), while diving ducks (genus *Aythya*) accounted for 5.0%, and geese (genera *Anser, Chen*, and *Branta*) 3.0% of the samples tested. Waterfowl (*Anatidae*) comprised 98.7% of samples, with 1.8% from non-migratory dabbling ducks (genus *Anas*). All samples were screened for avian influenza virus (AIV) by AIV-matrix real-time RT-PCR (rRT-PCR); all rRT-PCR positive samples (541) were processed for virus isolation as well as 4,473 rRT-PCR negative samples. Differences were observed in apparent prevalence estimates over the four years between virus isolation (0.5, 1.3, 3.9, and 0.7%) and rRT-PCR (5.9, 6.5, 11.2, and 5.5%). We isolated 138 AIVs, of which two were obtained from rRT-PCR negative samples. Unlike previous reports of seasonal variation in AIV prevalence, we documented differences in prevalence estimates among months using rRT-PCR only during 2008–2009 and by virus isolation only during 2006–2007 and 2007–2008.

Several of the AIV subtypes we identified are common in North America (e.g., H3, H4, and H6); H3N8 and H4N6 were the most common subtype combinations isolated. Similar to most surveillance studies, we found no significant difference in AIV infection based on host sex, but did find that juveniles were more likely to be positive for AIV than adults. We also documented that dabbling ducks were more likely to be positive for AIV than diving ducks, although not all dabbling ducks are equally likely to be positive. Molecular sequence analysis revealed no insertions of multiple basic amino acids at the cleavage site, which supported the identification of low pathogenic AIV. Phylogenetic anlyses performed on H5, H6, H7, N1, N2, N3, and N4 subtypes sequenced indicated similarity to other North American isolates with the exception of seven H6 which were more similar in amino acid translation to an isolate from Japan. In sum, this is the first multiyear study of avian influenza viruses on waterfowl wintering grounds of the Central Flyway, a historically understudied area of North America.

DEDICATION

To my husband, Michael, for his tireless love and support and Dr. Thomas Lester who vowed many years ago to make a dishwashing forklift driver a virologist and never gave up!

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First and foremost I thank God for seeing me through all of this and for richly blessing me with so many wonderful people and experiences; I could not have done any of this without Him and His abundant blessings.

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NOMENCLATURE

AI	Avian Influenza
AIV	Avian Influenza Virus
cRNA	Positive Strand RNA, Complementary RNA
HA	Hemagglutinin
ha	Hectares
HEDS	Highly Pathogenic Avian Influenza Virus Early Detection System
HI	Hemagglutination Inhibition
HP	Highly Pathogenic
HPAIV	Highly Pathogenic Avian Influenza Virus
LP	Low Pathogenic
LPAIV	Low Pathogenic Avian Influenza Virus
NA	Neuraminidase
M1	Matrix Protein
M2	Membrane Bound Ion Channel-like Protein
mRNA	Messenger-RNA
NEP/NS2	Nuclear Export Protein / Non-structural Protein 2
NI	Neuraminidase Inhibition
NP	Nucleocapsid Protein
NS1	Non-Structural Protein 1
NVSL	National Veterinary Services Laboratory

OIE	World Organization for Animal Health (Organisation Mondiale de la Santé Animale)
PA	Polymerase Acidic
PB1	Polymerase Basic 1
PB2	Polymerase Basic 2
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic Acid
RNP	Ribonucleoproteins
rRT-PCR	Real-time Reverse Transcription Polymerase Chain Reaction
TPB	Tryptose Phosphate Broth
vRNA	Viral RNA
WHO	World Health Organization

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1. Avian influenza viruses

Avian influenza (AI) was first described in 1878 by Edoardo Perroncito during an outbreak in poultry in northern Italy and was referred to as "fowl plague" (reviewed by Alexander, 2000; Capua and Alexander, 2004; Lupiani and Reddy, 2009). The disease was initially confused with the acute septicemic form of avian cholera; however, in 1880 based on clinical and pathological properties, the disease was shown to be different and was called *typhus exudatious gallinarium*. By 1901 the causative agent was shown to be an ultra-filterable agent (i.e. virus). However, the relationships among the classical fowl plague virus, and other less pathogenic viruses isolated from birds and the mammalian influenza A viruses were not demonstrated until 1955.

1.1.1. Classification of influenza viruses

Avian influenza viruses (AIV) are members of the *Influenzavirus A* genera which together with *Influenzavirus B* and *C*, *Thogotovirus*, and *Isavirus* form the family *Orthomyxoviridae* (Palese and Shaw, 2007). The *Orthomyxoviridae* family is characterized by a negative-sense, single-stranded, segmented RNA genome. While type A Influenza viruses can infect and cause disease in avian and mammalian species (Wright et al., 2007), types B and C are limited to human infections, although in rare

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This dissertation follows the style of Veterinary Microbiology.

cases type B and C viruses have been isolated from other species (Guo et al., 1983; Manuguerra and Hannoun, 1992; Osterhaus et al., 2000). Influenza A viruses are classified into subtypes based on two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) (Palese and Shaw, 2007).

Currently, there are 16 different HA subtypes (H1–16) and 9 different NA subtypes (N1–9), which can be found in up to 144 different combinations (Palese and Shaw, 2007). Depending on their virulence, AIVs are further classified as low pathogenic (LP) or highly pathogenic (HP) viruses (Capua and Alexander, 2004). Whereas LPAIV strains can cause asymptomatic to mild respiratory and enteric tract infections, HPAIV strain causes clinical illness and systemic disease and may cause mortality as high as 100%. Until now, only viruses from the H5 and H7 subtypes have been classified as HPAIV, but not all H5 and H7 viruses are HPAIV (Alexander, 2000).

1.1.2. Virion structure and composition

Influenza virus particles are pleomorphic varying in shape from spherical to filamentous, with a diameter of 80–120 nm and up to several microns in length (Mohanty and Dutta, 1981). Virions are composed of 0.8–1.0% RNA, 70% protein, 20% lipid, and 5.0 –8.0% carbohydrate (Ada and Perry, 1954; Frommhagen et al., 1959).

1.1.3. Genome structure and gene coding assignment

Type A influenza virus genome consists of eight RNA segments of negative polarity. These eight segments code for 10–12 viral proteins depending on the virus strain. The three largest segments encode for the three viral polymerase subunits, polymerase basic 1 and 2 (PB1 and PB2), and polymerase acidic (PA). The PB1

segment also encodes for a non-structural pro-apoptotic protein PB1-F2 and a newly discovered truncated form of PB1 (N40) (Wise et al., 2009). Two medium sized segments code for the structural glycoproteins (HA and NA), which form projections on the surface of the virus particle and are important antigenic determinants. The third medium sized segment codes for the viral nucleoprotein (NP), which together with the viral RNA and the polymerase complex, form eight ribonucleoproteins (RNP). The two smallest segments encode two proteins each. The M segment codes for the matrix protein (M1), which covers the inside of the viral envelope, and the membrane bound ion channel-like protein (M2). The NS segment encodes the non-structural protein 1 (NS1) and nuclear export protein (NEP or NS2).

1.1.4. Influenza virus replication

Attachment of the virus HA to the appropriate cellular receptor results in entry via receptor-mediated endocytosis (Kuiken et al., 2006; Lee et al., 2006). Once in the endosome, the virus-associated M2 protein allows the influx of H^+ ions into the virion, resulting in the disruption of protein–protein interactions, which results in the release of RNP free of the M1 protein. In addition, the reduction of pH within the endosome results in activation of the HA fusion properties resulting in fusion of virus and endosome membranes and release of viral contents into the cytoplasm. Subsequently, the viral RNPs are transported to the nucleus where transcription of viral mRNA and replication of the viral genome takes place (Gillim-Ross and Subbarao, 2006).

Once in the nucleus, three types of viral RNA are synthesized: viral mRNA (+ sense), viral genomic RNA (vRNA, - sense), and complementary viral RNA (cRNA, +

sense). All of these reactions are catalyzed by the viral RNA-dependent RNA polymerase complex, which consists of PB1, PB2, and PA proteins. The replication of virion RNA (viral genome) occurs in two steps: 1) synthesis of cRNA which are positive sense full-length copies of vRNA and serve as templates for generation of vRNA and 2) the copying of cRNA into vRNA. The synthesis of viral mRNA is dependent on host cellular RNA polymerase II activity because a 5' capped primer is required, which is obtained from newly synthesized host cell mRNA by the "cap snatching" mechanism carried out by the viral polymerase complex (Palese and Shaw, 2007).

During viral infection a dramatic shift from cellular to viral protein synthesis occurs. Translation occurs in the cytoplasm where newly synthesized mRNA associates with cellular ribosomes. The mRNAs coding for envelope proteins (M2, HA, and NA) are processed using the host cell's secretory pathway. These mRNAs are translated in the rough endoplasmic reticulum (RER) where the proteins undergo post-translational modification, pass through the Golgi undergoing additional modifications, and are then transported to the apical cell surface of polarized epithelial cells in vesicles. The mRNA coding for polymerase, capsid, matrix, and auxiliary proteins (M1, PB1, PB2, PA, NP, NS1 and NEP/NS2) are translated on free ribosomes and transported back into the nucleus where they associate with vRNA to form RNPs, which are exported into the cytoplasm and then transported to the apical surface of the infected cell where they associate with envelope proteins. Finally, once all components are present at the apical surface, the assembled virus particles bud out from the plasma membrane and are released from the cell surface and other viruses by cleavage of sialic acid by NA.

In most species, influenza viruses preferentially infect cells of the respiratory tract; however, in waterfowl AIVs replicate in cells of the intestinal tract. Viral attachment is accomplished by binding of the HA to the host cell receptor on the cell surface. The host cell receptor for type A influenza virus are glycans terminated by an α-2,6-linked sialic acid (SA α-2,6Gal) or an α-2,3-linked sialic acid (SA α-2,3Gal). The cell receptor for avian viruses is α-2,3-linked whereas that for humans and other mammals is α-2,6-linked. The predominant receptor in the human trachea and upper respiratory tract is the SA α-2,6Gal receptor. However, recent studies in humans have found avian-type receptors (SA α-2,3Gal) on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus as well as cells lining the alveolar wall (Ibricevic et al., 2006; Shinya et al., 2006; Thompson et al., 2006; van Riel et al., 2006). Therefore cells in the lower respiratory tract of humans have the appropriate receptors for binding of AIV strains. This explains why humans can be infected with AIV strains such as H5N1 without requiring adaptation for the SA α-2,6Gal receptor in an intermediate host such as pigs. More recent studies have detected both types of receptors in several other species, thereby identifying a number of hosts that may play a role in the evolution of new influenza subtypes with the ability to infect various species (Chaves et al., 2009; Hall et al., 2008).

1.1.5. The hemagglutinin (HA)

The HA is the major surface protein of influenza A viruses and is responsible for attachment of the virion to a susceptible cell. Pathogenicity is most often associated with the HA protein due to its binding activity to different surface receptors and proteases and

protease specificity for the cleavage site (Steinhauer, 1999). The HA is synthesized in the ER as a single polypeptide (HA0) and is subsequently cleaved by proteases into two disulfide-linked chains (HA1 and HA2). This cleavability of the hemagglutinin is essential for virus infectivity and a key factor in pathogenicity (Kuiken et al., 2006). In the case of LPAIV, HA0 is cleaved into HA1 and HA2 at the cell surface of a newly budding virion or on newly released virions by trypsin-like proteases produced by cells of the respiratory and enteric tract (Klenk and Rott, 1988). For HPAIV, post-translational modifications occur in the *trans* Golgi where cleavage by ubiquitous furin-like host proteases results in the cleavage of HA0 into HA1 and HA2, thereby resulting in expression of the active form of the HA on the surface of newly synthesized virions (Rott et al., 1995; Steinhauer, 1999). As a consequence, infection with HPAIV cause systemic infections that result in numerous vital organs being affected while LPAIV cause localized respiratory or intestinal infections.

1.1.6. The neuraminidase (NA)

The NA is the second major glycoprotein of influenza A viruses and is primarily responsible for the release of progeny virus from an infected cell (Palese and Shaw, 2007). The NA has an enzymatic activity that functions to remove sialic acid, which allows the release of virus from its host cell (Palese and Shaw, 2007). Interestingly, recent studies indicate additional roles for NA during the initial stages of infection, such as removing decoy receptors on mucins, cilia, and cellular glycocalix, all of which could impede virus access to functional receptors (Matrosovich et al., 2004). Due to the role of the NA in the release of progeny and the elucidation of its crystal structure, antiviral

drugs were developed that inhibit its function, oseltamivir (Tamiflu®) and zanamivir (Relenza®). The NA inhibitors function by blocking the NA's ability to cleave sialic acid residues, thereby inhibiting the release of progeny virions. NA inhibitors are most effective when administered within 48 hours of the onset of symptoms (Tullu, 2009). However, frequent usage of these antiviral drugs can result in the evolution of drug resistant strains, therefore the use in species other than humans is not common practice.

1.1.7. Influenza virus genetics

Due to the segmented nature of the influenza genome and the lack of proofreading of the viral polymerase, influenza viruses are able to change genetically fairly readily. The two methods by which new strains develop are through "genetic drift" and "genetic shift". Genetic drift is a consequence of point mutations due to the lack of proofreading of the viral RNA-dependent RNA polymerase. On the other hand, genetic shift occurs when a cell becomes infected with at least two different influenza viruses resulting in the exchange of segments (gene reassortment) between the two viruses. When genetic changes occur involving the HA or NA genes, the changes are commonly referred to as "antigenic drift" and "antigenic shift". Antigenic drift is the method by which epidemics typically arise, whereas antigenic shifts typically result in pandemics.

1.2. Ecology of type A influenza viruses

Species of the orders *Charadriiformes* (particularly gulls and shorebirds) and *Anseriformes* (waterfowl; ducks, geese, and swans) are considered the natural reservoir for all type A influenza viruses (Webster et al., 1992). All HA and NA subtypes have been identified in wild waterfowl, gulls, and shorebirds in a variety of different

combinations (Webster et al., 1992). On occasions, AIVs have been transmitted to other species such as domestic poultry, including chickens and turkeys (order *Galliformes*), humans, pigs, feral cats, and seals. To date, all influenza A viruses circulating in a variety of species can be traced back to an avian origin. The classical theory proposed by Webster et al., 1992 that pigs serve as "mixing vessels" and thus are the intermediate host for transmission of new strains of influenza to humans, remained unchallenged as the commonly accepted view until the emergence of H5N1 "bird flu". The direct transmission of H5N1 from avian hosts to human hosts resulted in approximately a 50% death rate in humans, thus challenging the classical theory of a required intermediate host, such as swine.

To date, the H5N1 "bird flu" has not yet adapted to transmit readily from human-to-human, which raises the questions regarding direct transmission efficiency of avian strains to humans without an intermediate mammalian host. The discovery of H5N1 direct transmission to humans from avian hosts resulted in increased surveillance efforts in a variety of species, including serologic surveillance in humans for antibodies to other avian strains of influenza virus. Not entirely surprising, these serologic studies revealed the presence of antibodies to avian strains in humans exposed to birds (Gill et al., 2006; Myers et al., 2007; Siembieda et al., 2008). Given that the first pandemic of the 21st century—H1N1 swine flu—originated with swine breathes continued life into the theory that swine serve as the intermediate "mixing vessel" for the emergence of influenza strains with significant human impact. Additionally, with the current pandemic being a

triple reassortant, in that it contains strains of avian, swine, and human origin, highlights the importance of the influenza gene pool in reservoir species, wild aquatic birds.

1.2.1. Wild aquatic birds and avian influenza viruses

In free-living birds, naturally occurring infections with LPAIV have been reported from at least 105 species of 26 different families (Olsen et al., 2006). In North America and Europe, all subtypes have been identified in wild ducks with the exception of H13 and H16, which have been primarily associated with shorebirds (Olsen et al., 2006). Interestingly, although all AIV subtypes have been identified in waterfowl, certain subtypes seem to be perpetuated in wild ducks more so than others. For example, in Canada (North America), H3, H4 and H6 as well as N2, N6, and N8 are the subtypes most commonly isolated (Krauss et al., 2004). Additionally, year-to-year variations have been reported in subtypes identified within bird populations, but few multi-year studies have been conducted (Krauss et al., 2004; Olsen et al., 2006; Wallensten et al., 2007).

HPAIV do not occur naturally in wild waterfowl; only after passage through domestic poultry do these viruses undergo genetic changes that result in HPAIV (Alexander, 2000). Prior to the emergence of HPAI H5N1, with the exception of A/tern/South Africa/1/61 (Becker, 1966), no clinical signs of disease or mortalities due to natural infections with AIV were reported in wild or domestic ducks (Alexander, 2000). Recently, however, one report identified an H5N2 virus circulating in healthy wild waterfowl with the molecular signature of a highly pathogenic virus (Gaidet et al., 2008). Since the emergence of HPAIV H5N1, these viruses have been transmitted from poultry to wild waterfowl and in outbreaks across Asia, the Middle East, Europe, and

Africa, wild birds now are suspected of playing a role as long-distance vectors of AIVs (Keawcharoen et al., 2008). The role of wild birds in the spread of HPAI, however, remains to be determined and more research is needed.

As stated by the WHO in a recent report (Organization, 2010), "Surveillance of influenza viruses in humans and animals should be strengthened to enable timely detection of epidemiological, clinical and virological changes" thus emphasizing the importance of continued surveillance in wild birds, humans, and other animal species. In order for the most information to be gained and progress to be made in understanding the ecology, natural history and evolution of these viruses as well as preparing a necessary response to influenza virus infections which could have a significant impact on human or animal health, timely sharing of information globally is essential (Capua and Alexander, 2008). Surveillance in reservoir species, such as wild aquatic birds, is of primary importance in order to monitor viruses that may become transmissible to other species.

1.2.2. Surveillance for avian influenza virus in wild aquatic birds

AIV surveillance in wild waterfowl can be separated into two phases, studies conducted prior to 2005 and those conducted thereafter. This separation is made based on increased awareness of AIVs and concern over the emergence of a pandemic strain of influenza related to the spread, persistence, and pathogenicity of H5N1 HPAI to both poultry and humans. Another factor influencing this separation was a shift in detection methods from primarily virus isolation to molecular-based methods that allowed a dramatic increase in testing capacity. Additionally, the shift in testing methods coupled with an increase in funding enabled more surveillance studies to be conducted. Thus,

over the last 5–6 years there has been a tremendous amount of surveillance data collected, but considerable inconsistency in test methods and sampling methods occurred, rendering comparisons across studies difficult (Cattoli and Capua, 2007). Further, the focus of the studies on HPAIV or LPAIV surveillance, which influences the testing methods and sample processing, contributed to the difficulty in comparisons across studies.

Although the introduction of HPAI into commercial poultry has enormous economic impact, introduction of LPAI virus can also have a significant economic impact. Thus, preventing the introduction and adaptation of wild-bird origin AIV to non-commercial and commercial poultry is an efficient strategy for minimizing the economic impact of AIVs. In order to carry out this strategy, it is important that we understand the prevalence, ecology, evolution, and molecular markers associated with interspecies transmission of AIV isolates in wild birds.

1.2.2.1. Global avian influenza surveillance in wild aquatic birds, in brief

Overlapping migratory routes worldwide provide an environment for the exchange of AIVs and for genetic reassortment to occur that could result in the spread of new AIV strains across continents. Although similar subtypes have been identified across different continents, phylogenetic analysis of viral genomes separate them into two distinct lineages—North American and Eurasian—indicating different selection pressures within populations of each region (Dugan et al., 2008; Krauss et al., 2007). Interestingly, a key concept in influenza virus evolution is that viruses associated with wild aquatic birds have reached an evolutionary stasis in that low rates of evolutionary

changes occur, particularly at amino acid-changing sites (Webster et al., 1992). Recent studies, however, have indicated that contrary to this popular belief of evolutionary stasis, AIVs actually exhibit rapid evolutionary changes (>1 x 10^{-3} substitutions per site per year; (Chen and Holmes, 2006; Worobey, 2008). Additionally, patterns affecting genetic diversity of AIVs probably have more to do with time and space as well as circulation of multiple viral lineages in a single location than species diversity (Chen and Holmes, 2009). Thus the selection pressures for certain subtypes have less to do with the species present and more to do with the time of year, location, and number of different subtypes circulating within the region. However, these studies examining evolutionary influences on subtype selection are limited in that there are few multi-year surveillance studies conducted within the same region available for analysis.

1.2.2.1.1. Sweden

A four-year study (2002–2005) in Sweden conducted during spring (March–June) and fall (July–December) consisted of 4,800 individual samples from 60 species, with most samples (85.5%) collected from mallards (*Anas platyrhynchos*) (Wallensten et al., 2007). The test methods utilized in this study differed from previous research and demonstrate a shift in testing methods. All 4,800 samples were pooled in groups of five and tested for AIV by molecular assays targeting the matrix gene, individuals from positive pools then were retested and any positive individuals processed for virus isolation. The most common HA subtypes identified were H4, H6, and H7 and the most common NA subtypes identified were N2, N6, and N7. The most common HA and NA subtype combinations identified were H4N6, H7N7, and H6N2.

Another study conducted over an eight-year period (1998–2006) in The Netherlands and Sweden tested 36,809 samples collected from 323 different species of 18 orders (Munster et al., 2007). The testing methods were similar to those of Wallensten et al., (2007) in that samples were pooled and tested by molecular methods before proceeding to virus isolation with the molecular test positive samples (Munster et al., 2007). This study identified a wider variety of subtypes than did that of Wallensten et al. (2007), but with similar results. The most common HA subtypes identified were H6 and H4, followed by H7, H3, H11, H1, H1, H5, and H10 and the most common NA subtypes identified were N2 and N6, followed by N8, N7, N9, N3, and N1 (Munster et al., 2007). The most common HA and NA subtype combination identified was H4N6 followed by H7N7 and H6N2 (Munster et al., 2007). These two studies showed some similarities in that H4 and H6, N2 and N6, and H4N6, H7N7, and H6N2 were common and most predominant in both studies, which is not entirely surprising in that the area and timeframe of the studies overlapped (Munster et al., 2007; Wallensten et al., 2007).

1.2.2.1.2. Eastern Germany

In eastern Germany, a 12-year study (1977–1989) was conducted utilizing both tracheal and cloacal swabs processed for virus isolation and blood samples screened for antibodies from wild birds, domestic ducks, and sentinel ducks (Suss et al., 1994). From 72,000 samples, 3,800 type A influenza viruses were isolated and only 1,253 were able to be subtyped using classical hemagglutination and neuraminidase inhibition assays (HI and NI, respectively). The most common hemagglutinin subtypes identified were H1, H2, and H4 and the most common neuramindase subtypes identified were N1, N2, N3,

and N6. The most common HA and NA subtype combinations identified were H6N1, H4N6, H6N2, H7N7, H3N8, and H2N3.

1.2.2.1.3. Canada

One of the most thorough studies published from North America is a 26-year study conducted in Canada (1976-2001), which consisted of 13,466 cloacal swab samples from migrating ducks collected during a banding project at the end of breeding season when the birds were staging for migration as well as 4,266 fecal samples or cloacal swabs from shorebirds (Krauss et al., 2004). The primary method of virus detection was virus isolation with molecular techniques used only when isolates could not be subtyped using classical HI and NI assays (Krauss et al., 2004). Throughout this study, H3, H4, and H6 subtypes were most frequently isolated from ducks; with H1, H2, H7, H10, and H11 subtypes occurring less frequently while H5, H8, H9, and H12 were rarely isolated. The most common HA and NA subtype combinations were: H3N8, H6N2, and H4N6. Additionally, they observed a cyclic pattern of occurrence with some subtypes appearing about every two years. In sum, this study supports the contention that ducks may be reservoirs for only certain subtypes of influenza viruses.

1.2.2.2. Surveillance in wild aquatic birds for avian influenza in the United States

The recent emergence and spread of highly pathogenic H5N1 virus from Asia to Europe and Africa has resulted in increased surveillance efforts worldwide with a primary focus on H5 and H7 AIV in both migratory birds and commercial poultry. Among the surveillance programs established within the United States, the “Interagency National Early Detection System for Highly Pathogenic AI Virus in Wild Birds in the

United States" (HEDS) includes efforts by Federal, State, and local governments, as well as non-government organizations, universities, and other interest groups (Agriculture, 2006). The HEDS surveillance program has focused on those species thought to be responsible for the spread of the Asian H5N1 virus (e.g., dabbling ducks, light geese, dark geese, and swans) and in those areas where large numbers of migratory waterfowl originating from Asia, Alaska, and Canada stop or overwinter. This surveillance program is essential for the early detection of highly pathogenic H5 and H7 AIV; however, it does not address the characterization of other low pathogenic AIV, which is essential for understanding their ecology, evolution, and mechanisms of transmission to other species.

Four major flyways used by migratory birds occur in North America: Pacific, Central, Mississippi, and Atlantic (htpp://www.flyways.us). Three of the four flyways (Pacific, Mississippi, and Atlantic) are well represented in the literature addressing AIV surveillance (Krauss et al., 2004); however, data are limited for the Central flyway, particularly the Texas Gulf Coast wintering grounds (Hanson et al., 2005; Kocan et al., 1980). Approximately 90% of the waterfowl that use the Central flyway winter in Texas. Of these, approximately 10 million ducks and geese winter in wetlands throughout the state, whereas 1 to 3 million ducks and over a million geese winter along the Texas Gulf Coast (DU, 2008). Prior to the implementation of surveillance programs focused on detecting H5N1 HPAIV, few surveillance studies included migratory waterfowl on their wintering grounds or non-migratory waterfowl during winter; this is particularly true for the Texas–Louisiana Gulf Coast where most studies were limited to just a few waterfowl

species and were limited by time of year as well as number of years studied (Hanson et al., 2005; Stallknecht et al., 1991; Stallknecht et al., 1990). Although the U.S. Interagency Strategic Plan for the Early Detection of Highly Pathogenic Avian Influenza H5N1 has extensively sampled waterfowl across all flyways, the focus of the program is the detection of H5N1; thus only information pertaining to H5N1 is available (USGS website http://wildlifedisease.nbii.gov/ai/index.jsp).

1.2.2.2.1. United States, pre-2005

Surveillance studies of wild aquatic birds for AIV in the United States conducted prior to 2005 were frequently limited to one year of sampling. One study, however, addressed six years (1978–1983) and consisted of 1,560 samples from a variety of water bird species (Hinshaw et al., 1985). Various subtypes of avian influenza virus were isolated throughout the course of the study along with differences in frequency noted. For example H4N8, H4N2, H6N2, and H3N8 were most common, but other subtypes were identified during some years. Interestingly, during one year (1980) no influenza viruses were isolated even though 336 waterfowl were examined.

A study conducted in Pennsylvania, Virginia, and Maryland included a wide variety of species but only covered November 1983–May 1984 (Nettles et al., 1985). Of 4,132 samples collected in Pennsylvania, nine presumptive AIV isolates were obtained; four were unable to be typed, probably due to the limited reagents available at the time. In Virginia, no AIVs were isolated from 313 samples tested, whereas 14 AIVs were isolated from 1,511 samples collected in Maryland The primary focus of this study was

to identify the source of an H5N2 epizootic that occurred in Pennsylvania, New Jersey, Maryland, and Virginia during 1983–1984.

During a three-year study (1986-1988) of hunter-harvested waterfowl (October–November) in Ohio, 55 avian influenza viruses were isolated from 1,450 birds sampled and 23 different HA–NA combinations were identified (Slemons et al., 1991). Prevalence of AIV in the wild bird population varied from 3.6 to 7.8% between years. No single HA–NA combination was recovered during all three years, but seven different combinations were identified in two of the three seasons (H3N2, H3N8, H6N2, H11N2, H11N9, H12N5, and H3/4N6). Different hemagglutinins were more common during the different years; in 1986, H11 was predominant, whereas H3 was predominant in 1987 and H6 in 1988.

During the fall of 1990 in Pennsylvania, 27 AIV were isolated from 330 cloacal swab samples collected from hunter-harvested waterfowl and cage-captured waterfowl during the summer of 1991 (Alfonso et al., 1995). The most prevalent subtype combinations were H4N8 and H6N8, most of the isolates were from mallards and juveniles were more likely to be positive for AIV.

A study conducted on the wintering grounds of the Mississippi Flyway, Gulf Coast of Louisiana, included 1,389 cloacal and tracheal swabs from hunter-harvested waterfowl during two consecutive hunting seasons (September 1987–January 1988, September 1988–January 1989) (Stallknecht et al., 1990). H4N6 was the most common subtype identified during both years, representing 32% of isolates identified. Two other subtypes (H1N1 and H11N9) also were identified during both seasons, with only one or

two isolates identified during each year. Despite occurring in different regions and at different times, some similarities among the subtypes identified were observed such as the detection of H4 and H6 as well as N6 and N8 subtypes, which is consistent with reports concerning common subtypes in North America (Krauss et al., 2004).

1.2.2.2.2. United States, 2005–present

The primary focus for AIV surveillance since 2005 has been the detection of H5N1 HPAI. This change in focus coupled with changes in testing methods (i.e., molecular based assays as opposed to virus isolation) means there are few surveillance studies within North America that can be directly compared over time. Nonetheless, some of the findings have been consistent across surveillance studies and over time. These include juveniles being at greater risk of AIV infection than adults, little to no difference in AIV prevalence by sex, variation in subtypes circulating among years, some consistency regarding subtypes associated with host populations, and some host species being more susceptible to AIV infection than others. Newer technologies and improved assays associated with interest in H5N1 surveillance have allowed identification of previously undetermined subtypes. Previously, isolate identification relied on anti-sera specific identification, whereas molecular methods now allow for sequencing in the absence of specific anti-sera.

Since 2005 surveillance for AIV in wild water birds in the United States has increased dramatically, however the availability of multiyear studies remains limited and the focus has been detection of H5 and H7 subtypes. One study sampled 16,797 birds in Alaska (May 2006–March 2007) and detected AIV in 1.7% using real-time RT-PCR

(rRT-PCR) methodologies and 1.5% by virus isolation (Ip et al., 2008). Virus isolation was performed on all rRT-PCR positive samples as well as 74% of the rRT-PCR negative samples, but no information on subtypes other than H5N1 were reported.

Two recent studies reporting subtype information other than H5N1 for the Pacific flyway have been published (Dusek et al., 2009; Siembieda et al., 2010). One study's focus was still H5N1 and testing methods were different in that they pooled samples in groups of five, screened with molecular methods, and only performed virus isolation on samples testing positive for H5 or H7; therefore subtype information other than H5N1 was limited (Dusek et al., 2009). Subtype combinations H4N2, H4N3, H4N6, and H6N2 were identified, along with several H5 subtypes (H5N2, H5N3, and H5N9). The other study combined methodologies by both screening using molecular methods (rRT-PCR) and then performing virus isolation only on PCR-positive samples as well as performing virus isolation on a subset of samples regardless of rRT-PCR results (Siembieda et al., 2010). Several subtypes were identified and year-to-year variations observed. H4, H6, and H10 as well as N2 and N3 were identified during all three years, with other subtypes appearing and disappearing between years. A study testing cloacal swab samples collected from migratory hunter-harvested, nonmigratory, and nesting waterfowl as well as migratory shorebirds from wildlife refuges in Alabama, Georgia, and Florida identified seven AIV isolates during one hunting season (September 2006–February 2007) and 12 isolates from the following season, although at the time of publication not all samples for that season had been tested (Dormitorio et al., 2009). Subtypes identified during the first year of the study were H1N1 (5), H1N4, and H10N7; during the second

year H3N8 (8), H4N6 (2), and H4N8 (2) were identified. These subtypes are similar to those previously reported within North America and support seasonal variation in subtypes circulating within a region.

1.2.2.2.3. Central Flyway of North America

Due to the low prevalence of AIV in waterfowl wintering grounds, particularly in the Central Flyway, little information is available concerning the year-to-year prevalence of AIV in this region. Prior to the current study, no multiyear study had been conducted within the Central Flyway. Previously, only two surveillance studies had been published from birds sampled in the Central Flyway, and both were limited in time and species examined (Hanson et al., 2005; Kocan et al., 1980). The duration of the Oklahoma study was from the fall 1976 through spring 1977 and included free-ranging waterfowl as well as sentinel mallards (Kocan et al., 1980). These authors isolated nine influenza viruses from 346 free-ranging ducks, included H1N1 (7), H6N2 (1), and H1N2 (1) subtypes, primarily from free-ranging mallards (7) with one isolate from a green-winged teal (*A. crecca*) and one from an American wigeon (*A. americana*). The second study conducted in the Central Flyway occurred in February and August 2001 and February 2002 and was limited to a few species [teals (*A. crecca, A. cyanoptera, A. discors*), mottled duck (*A. fulvigula*), and northern pintail (*A. acuta*)(Hanson et al., 2005)]. In this study, 22 avian influenza viruses were isolated from 258 samples collected and consisted of a variety of different subtypes: H1N3 (2), H1N4 (2), H2N4 (4), H2N9 (1), H7N3 (7), H7N4 (3), and H8N4 (3) (Hanson et al., 2005). Most isolates (81.8%) were from blue-winged teal sampled in February and no isolates were obtained from the resident species,

the mottled duck. Both studies indicate the presence of AIV in waterfowl of the Central Flyway and a low prevalence within the populations studied. However, a significant gap in knowledge exists concerning AIV in the Central Flyway.

1.3. Conclusions and research needs

Few multi-year surveys of AIV prevalence conducted within the same location have been reported, particularly in areas of low AIV prevalence, such as waterfowl wintering grounds. In order to develop a more comprehensive understanding of the ecology, natural history, and evolution of influenza viruses, long-term surveillance studies are needed. This is particularly true in understudied areas. Long-term surveillance is even more important in areas where commercial poultry operations and migratory waterfowl stop-over or wintering areas overlap, such as the Texas Gulf Coast (Miller, 2007).

The purpose of this study is to increase our knowledge of the prevalence, ecology, and evolution of AIVs isolated from wild waterfowl in an understudied geographical area, the wintering grounds of the Central Flyway will be addressed. The overall objective of this project is to complete a multiyear study of avian influenza on waterfowl wintering grounds of the Central Flyway in order to better understand the natural history and ecology of AIV and to detect AIV subtypes of concern to both human and animal health. The specific objectives of this study are:

1) To sample waterfowl and other legally hunted game birds brought to hunter check stations located along the Texas mid-Gulf Coast, throughout four consecutive hunting seasons.

2) To test collected samples for the presence of AIVs utilizing two testing methods, virus isolation (gold standard) and real-time RT-PCR.

3) To determine differences in sex, age, or species with regards to prevalence of AIV.

4) To determine genome sequence variability on selected isolates obtained from samples collected throughout the study.

1.4. Study area

The study area consisted of four state owned/managed wildlife management areas (WMA) located along the mid-Gulf Coast of Texas. This region is characteristic of Gulf Prairies and Marshes physiographic region of Texas (Gould, 1962). Topographically, the region where the WMAs are found ranges from sea level to a few feet in elevation and consists of freshwater to brackish wetlands (Hatch et al., 1990). Average rainfall is 66–142.2 cm annually (higher amounts in the eastern portion of the region), and a peak in September with a secondary peak in May. On average there are 245–320 frost-free days per year. The major soils in the Gulf Prairies and Marshes are Vertisols and Entisols. Typical vegetation types include grasses such as cordgrass (*Spartina* spp.), big bluestem (*Schizachyrium scoparium*), and indiangrass (*Sorghastrum nutans*) and trees and shrubs such as live oak (*Quercus virginia*), post oak (*Q. stellata*), huisache (*Acacia smallii*), blackbrush (*A. rigidula*), and bushy sea-ox-eye (*Borrichia frutescens*). Characteristic wildlife in the area include white-tailed deer (*Odocoileus virginianus*), alligators (*Alligator mississippiensis*), coyotes (*Canis latrans*), feral swine (*Sus scrofa*), a variety of avian species such as various migratory wetland-associated

birds, and the non-migratory mottled duck; the remaining wintering grounds for endangered whooping Crane (*Grus americana*) also is in this region.

The study sites were the Justin Hurst WMA (formerly Peach Point) in Brazoria County (28°56'55"N, 095°26'17"W), Mad Island WMA in Matagorda County (28°39'46"N, 096°00'17"W), Guadalupe Delta WMA in Calhoun County (28°30'47"N, 096°48'45"W), and Matagorda Island in Calhoun County (28°19'50"N, 096°27'51"W) (Fig. 1). These study sites were chosen based on the presence of hunter check stations and proximity (within 4–5 hr driving distance) to the research laboratory located in College Station, Texas. The Justin Hurst WMA consists of approximately 4,856 ha and is representative of the Gulf Coast Prairies and Marshes ecoregion, maintaining brackish marshlands as well as freshwater impoundments (Gould, 1962; Hatch et al., 1990; www.tpwd.state.tx.us, accessed 30 April 2010). Mad Island WMA is 2,913 ha of fresh to brackish marshland with sparse brush and level coastal prairie. Guadalupe Delta WMA consists of four units covering approximately 3,000 ha of freshwater marshes and is a complex of natural and manmade wetlands that are subject to flooding from the Guadalupe River and its adjacent bayous. The shallowness of adjacent bays and the volume of freshwater the Guadalupe River discharges, contribute to the extremely low salinity in these bay systems as compared to other bay systems in Texas. Riparian areas along the numerous small bayous are lined with pecan (*Carya illinoinensis*), black willow (*Salix nigra*), American elm (*Ulmus americana*), hackberry (*Celtis* spp.), and green ash (*Fraxinus pennsylvanica*), and provide excellent forage area for neotropical songbirds. Matagorda Island consists of 22,940 ha offshore barrier island and bayside

marshes. Few feral swine are observed on this island due to an aggressive removal program. The study sites were affected in September 2005 by hurricane Rita and by hurricane Ike in September 2008. These disturbances set back vegetative succession, which results in productive marshes for waterfowl (Bellrose, 1978).

Fig. 1. Hunter-harvested survey sample site locations.
(Map created by Map and GIS Collections and Services, Texas A&M University Libraries Software used: ESRI ArcMap 9.3)

CHAPTER II

AVIAN INFLUENZA SURVEILLANCE IN HUNTER-HARVESTED WATERFOWL FROM THE GULF COAST OF TEXAS (NOVEMBER 2005-JANUARY 2006)*

2.1. Overview

The objectives of our study were to determine prevalence of avian influenza viruses (AIV) on wintering grounds on the Texas Gulf Coast and to compare real-time reverse transcriptase polymerase chain reaction (rRT-PCR) and virus isolation for detection of AIV in cloacal swabs from wild waterfowl. Cloacal swabs were collected from hunter-harvested waterfowl from November 2005–January 2006 at four wildlife management areas. Seven AIV were isolated from four species of ducks: green-winged teal (*Anas crecca*) in November; blue-winged teal (*A. discors*) in November; mottled duck (*A. fulvigula*) in December, and northern shoveler (*A. clypeata*) in January. Prevalence of AIV for each of these species during the sampling period was 1.4, 2.1, 5.9, and 0.6%, respectively. The AIV subtypes detected were H1N2, H1N4, H4N6, H6N2, and H10N7, all previously reported in North American waterfowl. Our study identified AIV subtypes not previously reported on the Texas Gulf Coast and provides baseline data for a multiyear surveillance project.

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2.2. Introduction

Wild waterfowl are considered the natural reservoir of type A influenza viruses (Webster et al., 1992). The migratory nature of many waterfowl species and the persistence of influenza in these populations present a vehicle for dissemination of influenza viruses globally. Understanding the migratory patterns of different waterfowl populations, as well as identifying influenza virus subtypes within these populations, is critical to our understanding of how influenza viruses persist in nature and evolve over time. With the increased concern regarding the spread of highly pathogenic avian influenza (HPAI) H5N1 viruses, and wild waterfowl considered a vehicle for dissemination of the virus, several international surveillance programs have been implemented in an effort to reduce the potential worldwide spread of Asian origin H5N1 virus. Real-time reverse-transcription polymerase chain reaction (rRT-PCR) for the matrix gene, a highly conserved and abundantly expressed gene in type A influenza virus, has been the primary tool utilized for wild waterfowl surveillance, although virus isolation has also been used (Wild Bird Plan: An Early Detection System for Highly Pathogenic H5N1 Avian Influenza in Wild Migratory Birds U.S. Interagency Strategic Plan http://www.usda.gov/wps/portal/usdahome?contentidonly=true&contentid=2006/03/0094.xml, WHO Manual on Animal Influenza Diagnosis and Surveillance WHO/CDS/CSR/NCS/2002.5). In many of these surveillance programs, any sample positive for type A influenza virus are further screened by rRT-PCR specifically

targeting H5 and H7 subtypes, the two most commonly associated with losses in poultry (Alexander, 2000).

Prior to the implementation of surveillance programs focused on identifying HPAI H5N1, most studies of the prevalence of influenza viruses within North America were concentrated in Alaska, Canada, the upper Midwestern USA, and the Northeastern USA, and were conducted primarily during the late summer to early fall when pre-migration staging occurs (Krauss et al., 2004; Webster et al., 1992). Few studies involved waterfowl on their wintering grounds or nonmigratory waterfowl during the winter, particularly along the Gulf Coast, and most of these studies were limited to a few species [teals (*Anas crecca, A. cyanoptera, A. discors*), gadwall (*A. strepera*), mottled duck (*A. fulvigula*), northern pintail (*A. acuta*), and mallard (*A. platyrhynchos*)] (Hanson et al., 2005; Stallknecht et al., 1991; Stallknecht et al., 1990). In order to develop a more comprehensive understanding of the ecology of influenza viruses in nature, more extensive studies are needed. The objectives of this study were 1) to determine the prevalence of AIV in both migratory and resident waterfowl along the Texas Gulf Coast, focusing on wintertime sampling, and 2) to compare real-time RT-PCR and virus isolation for the detection of avian influenza (AI) using cloacal swabs collected from wild waterfowl.

2.3. Materials and methods

2.3.1. Sample collection

Cloacal swabs were collected from hunter-harvested waterfowl collected during the 2005–2006 hunting season (November–January) at four state wildlife management

areas (WMA) along the Gulf Coast of Texas: Peach Point (now called the Justin Hurst) WMA in Brazoria County (28°56'55"N, 095°26'17"W), Mad Island WMA in Matagorda County (28°39'46"N, 096°00'17"W), Guadalupe Delta WMA in Calhoun County (28°30'47"N, 096°48'45"W), and Matagorda Island WMA in Calhoun County (28°19'50"N, 096°27'51"W). Trained personnel identified waterfowl species. Data from all four WMAs were combined for analysis. The sex and age of the waterfowl were not consistently recorded thus were excluded from analysis.

Trained personnel collected cloacal swabs within 6 h of harvest using sterile Dacron swabs (Fisher Scientific, Houston TX, USA) and placed them in 1.5 ml tryptose phosphate broth (TPB; Becton Dickinson NJ, USA) supplemented with antibiotics [penicillin G ($2x10^3$ U/ml), streptomycin (200 µg/ml), gentamicin (250 µg/ml), and amphotercin B ($2x10^3$ U/ml) (Sigma, St. Louis MO, USA)] (Rosenberger et al., 1974; WHO Manual on Animal Influenza Diagnosis and Surveillance WHO/CDS/CSR/NCS/2002.5). Samples were transported from the field on wet ice (<10 hours collection and transport time) and stored at -80ºC until processed.

2.3.2. Sample processing

Samples were pre-processed for virus isolation and rRT-PCR as follows: samples were thawed and vortexed, swabs discarded, and the remaining fluid centrifuged 1,500 × g for 10 min. The supernatant was then diluted 1:2 in TPB containing antibiotics as listed above, and 100 µl was dispensed into 96-well plates for RNA isolation. Diluted samples and 96-well plates were frozen at -80ºC until processed for virus isolation or rRT-PCR.

2.3.3. Real-time RT-PCR

For rRT-PCR, 96-well plates were thawed and RNA was extracted from the samples using the MagMax™-96 Viral RNA Isolation Kit (Ambion Austin, TX; Cat#AM1836) according to the manufacturer's instructions. Extracted RNA was transferred to nuclease-free 96-well plates for immediate use. rRT-PCR was performed using the AgPath-ID™ AIV-M Reagent Kit (Ambion, TX; Cat#AM1014), a one-step rRT-PCR for the detection of AI matrix gene RNA as per manufacturer's instructions, and an ABI 7900HT (Applied Biosystems, Inc., USA) thermocycler in a 384-well format using a 15µl final reaction volume. Primers and probe for the M gene, H5, and H7 were those previously described (Spackman et al., 2002).

2.3.4. Virus isolation

For virus isolation, diluted samples were thawed and 0.2 ml was inoculated via the allantoic route into two 9-day-old embryonated chicken eggs. Eggs were incubated at 37ºC for 72 h, amnio-allantoic fluid (AAF) was collected, and subsequently tested for hemagglutination (HA) activity. Fluids negative for HA activity were reinoculated into two 9-day-old embryonated chicken eggs. HA positive fluids were further analyzed for the presence of influenza virus by rRT-PCR and/or FluDetect® (Synbiotics Inc., USA). Fluids testing positive for influenza virus by rRT-PCR and/or FluDetect® were sent to the National Veterinary Services Laboratory, in Ames, IA, USA for typing via classical methods [hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests]. Isolates that could not be typed by classical methods were typed by sequencing RT-PCR amplified HA genome segments as previously described (Hoffmann et al., 2001).

2.4. Results and discussion

From November 2005 to January 2006, 1,460 waterfowl were sampled and 86 were positive for AIV by rRT-PCR. Twenty-three hemagglutinating agents were identified from 896 samples processed for virus isolation. Seven of the hemagglutinating agents (isolates) were determined to be AIV (Table 1) and the remaining 16 are presumed to be paramyxoviruses. Five of the seven (71.4%) AIV isolates were obtained on first passage and the other two (28.6%) upon second passage in embryonated chicken eggs. Of the influenza-positive samples, whether by rRT-PCR and/or virus isolation, none were positive for H5 or H7. We were unable to determine the hemagglutinin subtype from two isolates (one green-winged teal and one mottled duck) using conventional HI testing. These isolates were determined to be H1 by RT-PCR amplification and sequencing of the PCR products. Waterfowl species collected and areas sampled reflect hunters' choices and personnel available to collect swabs on sampling days. More dabbling ducks were sampled than diving ducks—1,270 dabbling ducks representing 13 species as opposed to 145 diving ducks representing 7 species (Table 1). Overall prevalence of AIV based on rRT-PCR and virus isolation was 5.9 and 0.8%, respectively. Prevalence for the four species (green-winged teal, blue-winged teal, mottled duck, and northern shoveler) from which viruses were isolated were 1.4, 2.1, 5.9, and 0.6%, respectively; and by rRT-PCR 8.5, 10.4, 7.1, and 7.3%, respectively (Table 1).

Table 1. Results for avian influenza virus (AIV) virus isolation and real-time RT-PCR from cloacal swabs obtained from waterfowl along the Texas Gulf Coast of Texas, USA

Species	rRT-PCR[A]	Virus Isolation[A]	Isolate[B]
American coot (*Fulica Americana*)	0 / 13	0 / 10	
American wigeon (*Anas americana*)	2 / 109 (1.8%)	0 / 83	
Bufflehead (*Bucephala albeola*)	0 / 1	0 / 0	
Canada goose (*Branta Canadensis*)	0 / 4	0 / 1	
Canvasback (*Aythya valisineria*)	0 / 7	0 / 7	
Common snipe (*Gallinago gallinago*)	0 / 1	0 / 0	
Black-belly tree duck (*Dendrocygna autumnalis*)	0 / 10	0 / 0	
Gadwall (*Anas strepera*)	16 / 340 (4.7%)	0 / 149	
Greater white-front goose (*Anser albifrons*)	1 / 10 (10.0%)	0 / 4	
Hooded merganser (*Lophodytes cucullatus*)	0 / 4	0 / 3	
Lesser scaup (*Aythya affinis*)	1 / 50 (2.0%)	0 / 19	
Mallard (*Anas platyrhynchos*)	1 / 4 (25.0%)	0 / 2	
Mottled duck (*Anas fulvigula*)	3 / 42 (7.1%)	1 / 17 (5.9%)	H1N4[C]
Northern pintail (*Anas acuta*)	1 / 38 (2.6%)	0 / 36	
Northern shoveler (*Anas clypeata*)	17 / 234 (7.3%)	1 / 158 (0.6%)	H10N7
Redhead (*Aythya amercana*)	1 / 47 (2.1%)	0 / 40	
Ring-necked duck (*Aythya collaris*)	1 / 27 (3.7%)	0 / 13	
Ross's goose (*Chen rossii*)	0 / 2	0 / 0	
Ruddy duck (*Oxyura jamaicensis*)	0 / 9	0 / 7	
Teal (unidentified)	2 / 38 (5.3%)	0 / 5	
Teal, blue-winged (*Anas discors*)	16 / 154 (10.4%)	2 / 96 (2.1%)	H4N6 (n=2)
Teal, cinnamon (*Anas cyanoptera*)	0 / 2	0 / 2	
Teal, green-winged (*Anas crecca*)	24 / 284 (8.5%)	3 / 218 (1.4%)	H4N6 H6N2 H1N2[C]
Sandhill crane (*Grus candensis*)	0 / 4	0 / 4	
Snow goose (*Chen eaerulescens*)	0 / 24	0 / 22	
Wood duck (*Aix sponsa*)	0 / 2	0 / 0	
Total	86 / 1460 (5.9%)	7 / 896 (0.8%)	

[A]Number positive / number tested (prevalence)

[B]Isolates typed by NVSL

[C]The hemagglutinin of these isolates could not be subtyped by HI test and thus were subtyped by sequencing.

Our data support previous reports that dabbling ducks have a higher prevalence of infection with influenza viruses than other birds, including diving ducks (Olsen et al., 2006). Our sampling probably was biased to some unknown degree in that the variety of ducks sampled was in part a reflection of hunter's choices and not simply the relative abundance of each species. By targeting hunter-harvested waterfowl, however, we were able to estimate the prevalence of various AI subtypes carried by waterfowl in the Gulf Coast of Texas to which humans are likely to be exposed. After all, hunters are much more likely to be in direct contact with waterfowl than most other humans living in this region.

The 0.8% prevalence of AIV based on virus isolation reported here is consistent with previous reports of 0.4 to 2.0% on duck wintering grounds in the southern USA (Olsen et al., 2006; Stallknecht et al., 1990). Hanson et al. (Hanson et al., 2005), during an earlier study conducted at Peach Point, Texas, reported an AIV prevalence of >10% which was considered unusually high by the authors. Perhaps the time of year and/or the year samples were collected could account for the discrepancy. Variables such as weather conditions and population densities could affect virus prevalence. Most samples collected by Hanson et al. (Hanson et al., 2005), were taken in February and included only a few species [teals (*Anas crecca, A. cyanoptera, A. discors*), mottled duck (*A. fulvigula*), and northern pintail (*A. acuta*)], while our study focused on the wintering months (November–January) and included many more species (Table 1). One other study, conducted on the Gulf Coast of Louisiana, reported similar results to ours (Stallknecht et al., 1990). These authors reported an overall prevalence of 2.0% in

November and 0.4% for December–January as compared to our 1.7% in November and 0.3% for December–January. Year-to-year and day-to-day variations in subtype and prevalence have been reported in other surveillance studies (Runstadler et al., 2007; Sharp et al., 1993).

The subtypes we identified are consistent with previous studies and are common North American subtypes. The H3, H4, and H6 are considered the most common, while H1, H2, H7, H10, and H11 are less common. Krauss et al. (2004), in a 26-year study of wild ducks in Canada, found that the most frequent subtypes isolated from ducks were H3N8 (22.8%), H6N4 (20.8%), and H4N6 (12.5%). In our study, 3 of the 7 isolates were H4N6; interestingly, this subtype had previously been reported in Louisiana but not in Texas (Hanson et al., 2005; Stallknecht et al., 1990).

The discrepancy between rRT-PCR results and virus isolation is not surprising. It is generally accepted that rRT-PCR is more sensitive than virus isolation because of its ability to detect both infectious and non-infectious viral particles (Krafft et al., 2005; Runstadler et al., 2007). It is possible the two freeze-thaw cycles our samples underwent, might have lead to negative isolation results for samples with low levels of virus. Several samples, however, underwent additional freeze-thaw cycles for re-isolation attempts without problems, so we expect two freeze-thaw cycles to have minimal effect. Another possible explanation for this discrepancy could be the length of time between hunter-harvest and sampling. During the teal season hunters typically leave their blinds and pass through the check station within 2 hours of bagging their teal; whereas, during the regular waterfowl season, most hunters remain in their blinds for the duration of hunting

hours (sometimes up to 6 h post shooting time) (Ferro, personal observations). It is also possible that the use of embryonated chicken eggs for virus isolation limits the isolation to those viruses capable of replicating in this system. The use of commercial eggs as opposed to specific pathogen free eggs could be a concern; however, due to the extensive AI surveillance in U.S. poultry and the lack of vaccination of U.S. poultry flocks, the concern is minimal.

Much information is available on the prevalence of AIV in ducks on pre-migration staging grounds (Krauss et al., 2004; Sharp et al., 1993). With current technology, large-scale sequence analysis of AIV isolates is possible and can provide valuable information about how influenza viruses persist in nature (Hatchette et al., 2004; Obenauer et al., 2006). Further studies involving the molecular characterization and comparison of the same influenza virus subtype from different regions along a flyway will provide significant information regarding what changes within AIV occur in nature. Similarly, studies following target species (those identified as having a high prevalence), throughout their migration, could provide valuable information regarding persistence of AIV in these species. Finally, studies covering consecutive years in the same wintering grounds will help us understand the ecology and evolution of influenza viruses and how these viruses persist in nature over winter. This study contributes to the knowledge base of influenza virus prevalence on waterfowl wintering grounds in Texas and provides baseline information for a multi-year surveillance project. Information gained over the next few years will assist in the elucidation of subtype prevalence,

evolution, and persistence of AI in wild waterfowl, including migratory and non-migratory species, on wintering grounds.

CHAPTER III

MULTIYEAR SURVEILLANCE FOR AVIAN INFLUENZA VIRUS IN HUNTER-HARVESTED WATERFOWL FROM THE WINTERING GROUNDS OF THE TEXAS MID-GULF COAST (SEPTEMBER 2006-JANUARY 2008)*

3.1. Overview

Of 5,363 hunter-harvested migratory and resident waterfowl and wetland-associated game birds sampled during three consecutive hunting seasons (September 2006–January 2007 through September 2008–January 2009), real-time RT-PCR detected influenza matrix gene sequence in 8.48% of the samples, H5 gene sequence in 0.69%, and H7 gene sequence in 0.60%. Virus isolation yielded 136 type A influenza viruses including all nine neuraminidase (N1-9) and nine hemagglutinin (H1-7, 10, and 11) subtypes. Low pathogenic H7 viruses were isolated during January, September, and November of 2007 and January 2008 (no.=13) while low pathogenic H5 viruses were isolated during November and December 2007 (no.=8). Data herein show blue-winged teal, green-winged teal, and northern shovelers are high prevalence species for AIV and juveniles are more likely to be infected than adults. This is the first multi-year study of wintering waterfowl populations on the coast of the Central Flyway, a historically understudied area of North America.

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*Ferro, P.J., C.M Budke, M.J. Peterson, D. Cox, E. Roltsch, T. Merendino, M. Nelson, B. Lupiani (2010) Multiyear surveillance of avian influenza virus in hunter-harvested waterfowl from the wintering grounds of the Texas mid–Gulf Coast. *Emerging Infectious Diseases,* accepted 30 April 2010.

3.2. Introduction

Wild waterfowl, primarily species in the orders Charadriiformes and Anseriformes (Webster et al., 1992), are natural reservoirs for type A influenza viruses, which are occasionally transmitted to other species, including humans, poultry and swine, resulting in subclinical to highly pathogenic diseases. Two subtypes have been most frequently associated with high pathogenicity in poultry, H5 and H7, and are of considerable interest to the poultry industry, and researchers studying avian influenza viruses (AIV) (Dusek et al., 2009; Munster et al., 2005; Reperant et al., 2009). The migratory nature of many waterfowl species and the persistence of AIV in these populations present a potential vehicle for global dissemination of influenza viruses as well as a constant source of viruses and genetic material for new pandemic strains. Preventing the introduction and adaptation of wild-bird origin AIV to other susceptible species is considered an efficient strategy for minimizing the impact of AIV on global health and economy (Agriculture, 2006; Capua and Alexander, 2006). Thus, surveillance in reservoir species is crucial in order to identify viruses and gene pools with inter- and intra-species transmission potential.

Four major flyways used by migratory birds occur in North America: Pacific, Central, Mississippi, and Atlantic (htpp://www.flyways.us). Three of the four flyways (Pacific, Mississippi, and Atlantic) are well represented in the literature addressing AIV surveillance (summarized in (Krauss et al., 2004); however, data are limited for the Central flyway (Hanson et al., 2005; Kocan et al., 1980). Approximately 90% of the waterfowl that use the Central flyway winter in Texas. Of these, about 10 million ducks

and geese winter in wetlands throughout the state, whereas 1 to 3 million ducks and over a million geese winter along the Texas Gulf Coast (DU, 2008). Prior to the implementation of surveillance programs focused on detecting H5N1 highly pathogenic avian influenza virus (HPAIV), few surveillance studies included migratory waterfowl on their wintering grounds or non-migratory waterfowl during winter; this is particularly true for the Texas–Louisiana Gulf Coast where most studies were limited to just a few waterfowl species and limited by time of year as well as number of years studied (Hanson et al., 2005; Stallknecht et al., 1991; Stallknecht et al., 1990). Although the U.S. Interagency Strategic Plan for the Early Detection of Highly Pathogenic Avian Influenza H5N1 has extensively sampled waterfowl across all flyways, the focus of the program is the detection of H5N1; thus only information pertaining to H5N1 is publicly available (NBII, 2007). In order to develop a more comprehensive understanding of the ecology, natural history, and evolution of influenza viruses, long-term surveillance studies are needed, particularly those addressing waterfowl in understudied areas such as wintering grounds. Long-term surveillance is even more important in areas where commercial poultry operations and migratory waterfowl stop-over or wintering areas overlap (Miller, 2007).

We recently reported AIV prevalence, as determined by real-time RT-PCR (rRT-PCR) and virus isolation, from a multiyear surveillance project (September 2005–January 2009) targeting hunter-harvested waterfowl in the mid-Texas coast region (Ferro et al., 2010). We found little variation in overall AIV prevalence within or between seasons with the exception of one season (2007–2008) where the overall prevalence was

higher (Ferro et al., 2010). A recent report by Siembada et al. (2009) identified hunters as the human population most at risk of exposure to AIV, therefore by targeting hunter-harvested waterfowl and other wetland-associated game birds, we were able to isolate AIV to which humans are most likely to be exposed (Siembieda et al., 2008). The objectives of the current study were 1) to determine subtype diversity of AIV in both migratory and resident waterfowl populations (mostly ducks and geese) to which humans may be exposed and 2) to compare prevalence and subtype diversity of AIV among species, age, and sex focusing on the Texas mid-Gulf Coast region during early fall and winter, coinciding with the regional hunting season.

3.3. Materials and methods

3.3.1. Sample collection

Cloacal swab samples were collected from hunter-harvested waterfowl (Bellrose, 1978) and other wetland-associated game birds (Tacha and Braun, 1994) during three consecutive hunting seasons (September 2006–January 2007, September 2007–January 2008, and September 2008–January 2009) at four state wildlife management areas (WMA) along the Gulf Coast of Texas: Justin Hurst WMA in Brazoria County, Mad Island WMA in Matagorda County, Guadalupe Delta WMA in Calhoun County, and Matagorda Island WMA in Calhoun County (Fig. 1). Trained field personnel identified species, sex, and age (when possible) based on plumage (Braun, 2005). Age was recorded as adult reflecting after-hatch-year and juvenile reflecting hatch-year birds. Waterfowl species and areas sampled reflect hunters' choices and personnel available to collect swabs on sampling days. Data from all four WMAs were combined for analysis.

3.3.2. Avian influenza virus testing

All samples were collected, processed, and tested as previously described (Chapter II). Briefly, all samples (5,363) were screened for AIV by AIV-matrix rRT-PCR, and virus isolation was performed on all rRT-PCR positive samples (455) as well as 3,664 rRT-PCR negative samples. Additionally, all rRT-PCR positive samples (455) were screened for H5 and H7 subtype viruses by rRT-PCR using the AgPath-ID^{TM} One-Step RT-PCR Kit (Ambion, Inc., Austin, TX, USA) and an ABI 7500Fast Real-time PCR System (Applied Biosystems, Inc., Foster City, CA, USA) in a 25µl final reaction volume. Primers and probes for the M and H5 (Spackman et al., 2003; Spackman et al., 2002) and H7 (Spackman et al., 2008; Spackman et al., 2002) were those previously described. All AIV isolates were submitted to the National Veterinary Services Laboratory (NVSL; Ames, IA, USA) for subtyping by hemagglutination and neuraminidase inhibition tests and screening for the presence of the N1 gene by rRT-PCR. Additionally, all H5 and H7 isolates were pathotyped at the NVSL by analysis of the amino acid sequence at the hemagglutinin protein cleavage site.

3.3.3. Statistical analysis

We previously documented that prevalence estimates calculated on virus isolation following a positive AIV-matrix rRT-PCR provided results nearly identical to those obtained by performing both tests in parallel (Chapter IV); for this reason we calculated apparent prevalence by dividing the number of virus isolation positive samples (following a positive rRT-PCR result) by the total number of samples collected and tested using rRT-PCR (Ferro et al., 2010).

Pearson's chi-square analyses were used to evaluate differences in AIV infected proportion by sex (drake vs. hen), age (adult vs. juvenile), species of waterfowl, and hunting season of collection (2006–2007, 2007–2008, and 2008–2009). Fisher's exact test was used instead of chi-square when one or more cells was expected to have a frequency of $\leq$5. A p-value <0.05 was considered statistically significant. Wald 95% confidence intervals were calculated for all AIV infected proportions (i.e., sex, age, species).

A multivariate main effects logistic regression model was also constructed to assess differences in avian influenzas virus detection using rRT-PCR by age, sex, and bird species. Species were categorized into blue-winged teal, green-winged teal, gadwall, northern shoveler, and "other species". The four species-specific categories were chosen as they represented the largest numbers of tested birds. Sample records missing rRT-PCR results or age, sex, or species data were removed from this analysis. All analyses were performed using Intercooled Stata version 9 (Stata Corp., College Station, TX, USA).

3.4. Results

3.4.1. Sampling overview

A total of 5,363 cloacal swab samples were collected from 33 different potential host species, including a variety of waterfowl and other wetland-associated game birds (Tables 2–4) during three consecutive hunting seasons (September 2006–January 2007: 2,171; September 2007–January 2008: 2,424; and September 2008–January 2009: 768). The majority of samples (58.51%, no.=3,138) were from teal (*Anas discors* and *A.*

crecca), followed by northern shovelers (*A. clypeata*; 13.11%, no.=703), gadwall (*A. strepera*; 8.15%, no.=437), and American wigeon (*A. americana*; 4.44%, n=238), with the remaining samples 15.79% (no.=847) from a variety of other species (Tables 2–4). Adults accounted for 51.45% (no.=2,759) of samples, whereas 28.04% (no.=1,504) were collected from juveniles, and 20.51% (no.=1,100) from individuals of undetermined age. Additionally, 45.59% (no.=2,445) of samples were from drakes, 42.18% (no.=2,262) from hens, and 12.23% (no.=656) were from birds of undetermined sex.

3.4.2. Subtype prevalences

Of 4,119 samples processed for virus isolation, 136 type A influenza viruses were isolated. All nine neuraminidase subtypes (N1–9) were isolated, whereas only nine of the sixteen different hemagglutinin subtypes (H1–7, 10, and 11) were isolated (Fig. 2). Thirty-two different hemagglutinin and neuraminidase combinations were identified (Table 5), and for eight of the isolates either the hemagglutinin (no.=7) or neuraminidase (no.=1) was not identified.

Table 2. Apparent prevalence of avian influenza virus in various waterfowl and wetland-associated gamebird species as determined by virus isolation and real-time RT-PCR from cloacal swabs collected from hunter-harvested waterfowl, Texas mid-Gulf Coast, USA September 2006–January 2007.

Species	No.	rRT-PCR [A]	Virus Isolation [A]	Isolate [B]
American wigeon (*Anas americana*)	171	4 (2.34%)	0	
Fulvous whistling duck (*Dendrocygna bicolor*)	18	2 (11.11%)	1 (5.56%)	H6N1
Lesser scaup (*Aythya affinis*)	60	1 (1.67%)	1 (1.67%)	H10N7
Mallard (*Anas platyrhynchos*)	5	1 (20.00%)	0	
Mottled duck (*Anas fulvigula*)	33	2 (6.06%)	1 (3.03%)	H6N5
Northern pintail (*Anas acuta*)	72	5 (6.94%)	0	
Northern shoveler (*Anas clypeata*)	360	23 (6.39%)	4 (1.11%)	H2N9 H3N8 H4N2 H4N6 H4N8
Redhead (*Aythya amercana*)	51	2 (3.92%)	0	
Ring-necked duck (*Aythya collaris*)	35	1 (2.86%)	0	
Ruddy duck (*Oxyura jamaicensis*)	31	2 (6.45%)	0	
Teal, blue-winged (*Anas discors*)	610	65 (10.66%)	15 (2.46%)	H1N1 H2N9 H3N6 H3N8 (6) H4N2 H4N6 H4N8 H6N1 (3) H6N1/4 H6N5 H6N6 H6N8
Teal, green-winged (*Anas crecca*)	358	31 (8.66%)	5 (1.40%)	H1N1 H6N2 H7N3 H10N7 H11N3
Snow goose (*Chen eaerulescens*)	46	2 (4.35%)	0	
Total [C]	2171	141 (6.49%)	29 (1.34%)	

[A]Number positive (apparent prevalence). Numbers for virus isolation are following an rRT-PCR result.
[B]Isolates typed by NVSL. Included are virus isolates that were rRT-PCR negative on the original sample.
[C]Other species sampled that were negative for AI by rRT-PCR and VI, number sampled: American coot (*Fulica americana*), 25; Black-bellied whistling duck (*Dendrocygna autumnalis*), 6; Bufflehead (*Bucephala albeola*), 2; Canvasback (*Aythya valisineria*), 16; Cinnamon teal (*Anas cyanoptera*), 1; Common goldeneye (*Bucephala clangula*), 1;Common moorhen (*Gallinula chloropus*), 1; Common snipe (*Gallinago gallinago*), 1; Gadwall (*Anas strepera*), 247; Greater white-front goose (*Anser albifrons*), 3; Hooded merganser (*Laphodytes cucullatus*), 11; Mottled duck x Mallard hybrid (*Anas fulvigula x Anas platyrhynchos*), 1; Ross's goose (*Anser albifrons*), 2; Sandhill crane (*Grus candensis*), 1; Wood duck (*Aix sponsa*), 1; and unidentified teal, 2.

Table 3. Apparent prevalence of avian influenza virus in various waterfowl and wetland-associated game bird species as determined by virus isolation and real-time RT-PCR from cloacal swabs collected from hunter-harvested waterfowl, Texas mid-Gulf Coast, USA September 2007–January 2008.

Species	No.	rRT-PCR[A]	Virus Isolation[A]	Isolate[B]
American wigeon (*Anas americana*)	51	8 (15.69%)	0	
Fulvous whistling duck (*Dendrocygna bicolor*)	14	1 (7.14%)	0	
Gadwall (*Anas strepera*)	160	10 (6.25%)	1 (0.63%)	H6N1
Mottled duck (*Anas fulvigula*)	26	1 (3.85%)	0	
Mottled duck x Mallard (*Anas fulvigula x platyrhynchos*)	2	1[C]	1[C]	H6N8
Northern pintail (*Anas acuta*)	62	13 (20.97%)	3 (4.84%)	H4N8 H10N3 H10N3/7
Northern shoveler (*Anas clypeata*)	239	38 (15.90%)	10 (4.18%)	H4N2 H5N2 (2) H5N3 H6N2 (2) H10N2 H10N7 H11N9 (2)
Ring-necked duck (*Aythya collaris*)	17	1 (5.88%)	0	
Ruddy duck (*Oxyura jamaicensis*)	36	2 (5.56%)	1 (2.78%)	H2N3
Teal, blue-winged (*Anas discors*)	1213	155 (12.78%)	72 (5.94%)	H1N1 (2) H2N8 H3N4 H3N6 (2) H3N8 (9) H4N1 H4N6 (17) H4N8 (6) H5N2 (2) H5N3 (2) H6N1 H7N1 H7N1/4 H7N4 H7N7 (5) H10N? H10N3 (2) H10N3/7 H10N7 (7) H11N9 (3)
Teal, cinnamon (*Anas cyanoptera*)	2	1[C]	1[C]	H7N3
Teal, green-winged (*Anas crecca*)	464	38 (8.19%)	6 (1.29%)	H5N2 H7N1/4 H7N3 H10N3 H10N3/7 H10N7 H11N9
Snow goose (*Chen eaerulescens*)	43	3 (6.98%)	0	
Total[D]	2424	272 (11.22%)	95 (3.92%)	

[A]Number positive (apparent prevalence) Numbers and apparent prevalence for virus isolation are following an rRT-PCR result.
[B]Isolates typed by NVSL, 7 isolates were confirmed AI but unable to be subtyped. Included are virus isolates that were rRT-PCR negative on the original sample.
[C]Apparent prevalence not calculated due to the small sample size.
[D]Other species sampled that were negative for AI by rRT-PCR and VI, number sampled: American coot (*Fulica americana*), 27; Black-bellied whistling duck (*Dendrocygna autumnalis*), 9; Canvasback (*Aythya valisineria*), 3; Common snipe (*Gallinago gallinago*), 2; Greater white-front goose (*Anser albifrons*), 3; Hooded merganser (*Laphodytes cucullatus*), 3; Least grebe (*Tachybaptus dominicus*), 1; Lesser Scaup (*Aythya affinis*), 26; Mallard (*Anas platyrhynchos*), 4; Redhead (*Aythya amercana*), 3; Ross's goose (*Anser albifrons*), 5; Sandhill crane (*Grus candensis*), 8; and unidentified teal, 1.

Table 4. Apparent prevalence of avian influenza virus in various waterfowl and wetland-associated game bird species as determined by virus isolation and real-time RT-PCR from cloacal swabs collected from hunter-harvested waterfowl, Texas mid-Gulf Coast, USA September 2008–January 2009.

Species	No.	rRT-PCR[A]	Virus Isolation[A]	Isolate[B]
American wigeon (*Anas americana*)	16	1 (6.25%)	0	
Northern pintail (*Anas acuta*)	28	2 (7.14%)	1 (3.57%)	H4N6
Northern shoveler (*Anas clypeata*)	104	5 (4.81%)	1 (0.96%)	H7N2
Teal, blue-winged (*Anas discors*)	176	21 (11.93%)	3 (1.70%)	H4N6 H4N8 (2)
Teal, green-winged (*Anas crecca*)	314	13 (4.14%)	0	
Total[C]	768	42 (5.47%)	5 (0.65%)	

[A]Number positive (apparent prevalence)
[B]Isolates typed by NVSL
[C]Other species sampled that were negative for AI by rRT-PCR and VI, number sampled: Common ground dove (*Columbina passerina*), 1; Gadwall (*Anas strepera*), 30; Greater white-front goose (*Anser albifrons*), 6; Hooded merganser (*Laphodytes cucullatus*), 4; Lesser Scaup (*Aythya affinis*), 1; Mallard (*Anas platyrhynchos*), 4; Mottled duck (*Anas fulvigla*), 23; Mottled duck x Mallard hybrid (*Anas fulvigula x Anas platyrhynchos*), 1; Ring-necked duck (*Aythya collaris*), 1; Ross's goose (*Anser albifrons*), 1; Sandhill crane (*Grus candensis*), 1; Snow goose (*Chen eaerulescens*), 56; and Wood duck (*Aix sponsa*), 1.

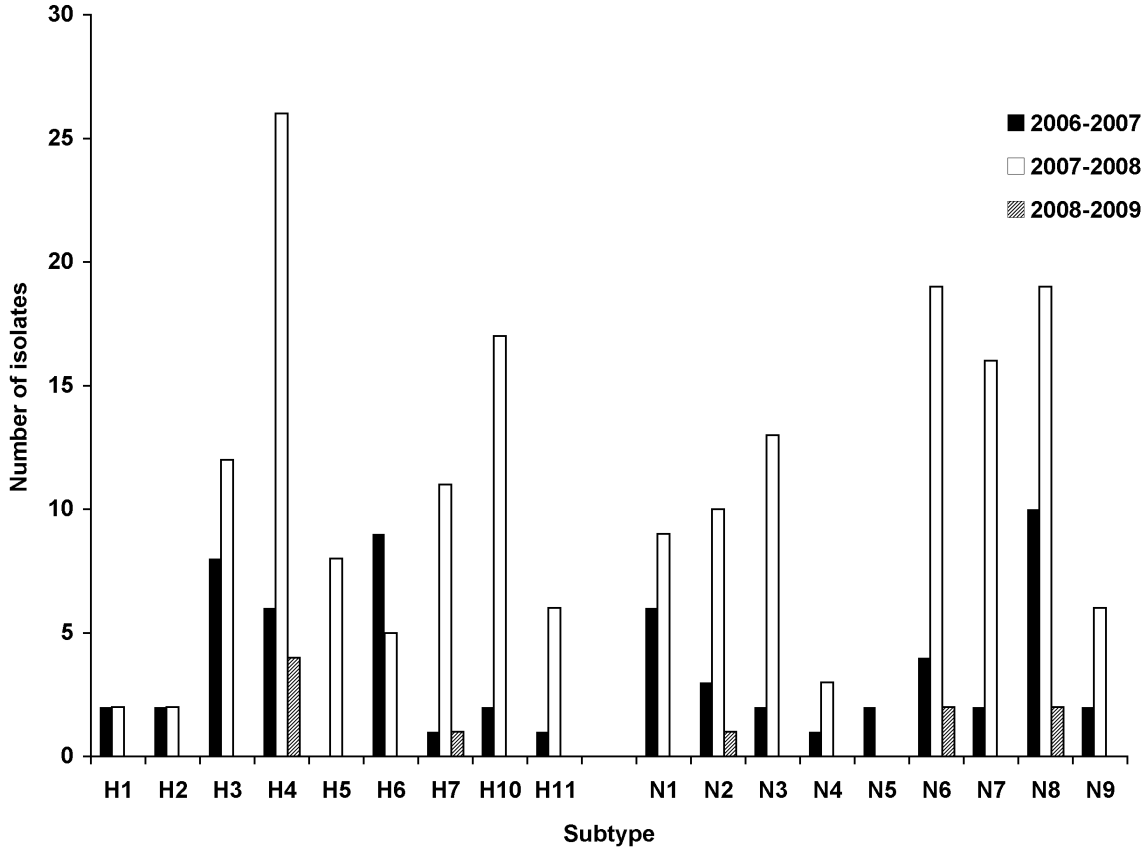

Fig. 2. Comparison of hemagglutinin (H) and neuraminidase (N) subtypes of AIV isolated during three consecutive hunting seasons, 2006–2007 through 2008–2009, Texas mid-Gulf coast, USA.

Table 5. Hemagglutinin and neuramindase combinations for all avian influenza viruses isolated from hunter-harvested waterfowl, Texas Gulf Coast, USA (September 2006–January 2007 through September 2008–January 2009).

			Hemagglutinin (HA)[A]									
		Hunt season	1	2	3	4	5	6	7	10	11	Total
Neuraminidase (NA)	1	2006–2007	2					5				7
		2007–2008	2			1		2	3			8
	2	2006–2007				2		1				3
		2007–2008				1	5	2		1		9
		2008–2009							1			1
	3	2006–2007							1		1	2
		2007–2008		1			3		2	7		13
	4	2006–2007										0
		2007–2008			1				1			2
	5	2006–2007						2				2
		2007–2008										0
	6	2006–2007			1	2		1				4
		2007–2008			2	17						19
		2008–2009				2						2
	7	2006–2007								2		2
		2007–2008							5	9		14
	8	2006–2007			7	2		1				10
		2007–2008		1	9	7		1				18
		2008–2009				2						2
	9	2006–2007		2								2
		2007–2008									6	6
	Total	2006–2007	2	2	8	6	0	10	1	2	1	32
		2007–2008	2	2	12	26	8	5	11	17	6	89[B]
		2008–2009	0	0	0	4	0	0	1	0	0	5
		Total	4	4	20	36	8	15	13	19	7	126

[A]No H8, H9, nor H12–16 were identified in this study.
[B]8 isolates not recorded due to an inability to subtype the hemagglutinin and/or neuraminidase. All isolates included regardless of rRT-PCR result.

The most frequently identified hemagglutinin subtype during September 2006–January 2007 were H3 and H6 (25.00%, no.=8 and 28.12%, no.=9, respectively), whereas for September 2007–January 2008 H4 and H10 were predominant (26.80%, no.=26 and 17.53%, no.=17, respectively), with H4 (80.00%, no.=4) remaining predominant the following year (September 2008–January 2009). With respect to neuraminidase subtypes, N1 and N8 were most common the first season (18.75%, no.=8 and 31.25%, no.=10, respectively), whereas N6, N7, and N8 (19.59%, no.=19; 16.49%, no.=16 and 19.59% no.=19, respectively) were predominant the second season (September 2007–January 2008), with N6 and N8 (40.00%, no.=2 each) remaining predominant the third season (September 2008–January 2009) The most frequent hemagglutinin and neuraminidase combinations identified during the first season were H3N8 (no.=7) and H6N1 (no.=4), whereas H4N6 (no.=17), H3N8 (no.=9), and H10N7 (no.=9) were the most common combinations identified the second season, and H4N6 (no.=2) and H4N8 (no.=2) were most common the third season (Table 5).

H7 subtypes were identified by rRT-PCR during all three hunting seasons (September–January 2006–2007 through 2008–2009, no.=2, 28, and 2, respectively) (Spackman et al., 2008). Additionally, H5 subtypes were detected by rRT-PCR all three seasons (no.=14, 21, and 2, respectively). Yet, H5 viruses were isolated only during the 2007–08 hunting season, whereas H7 viruses were isolated during all three hunting seasons (Tables 6 and 7). All H5 and H7 viruses were determined to be low-pathogenic AIVs by analysis of the amino acid sequence at the hemagglutinin protein cleavage site.

Table 6. Subtypes of avian influenza viruses isolated in the fall (September and November) from select species during three consecutive hunting seasons, September 2006–January 2007 through September 2008–January 2009, Texas mid-Gulf Coast, USA.

Species[A]	September[B]			November		
	2006	2007	2008	2006	2007	2008
Fulvous whistling duck (*Dendrocygna bicolor*)				H6N1		
Mottled duck x Mallard (*Anas fulvigula x platyrhynchos*)					H6N8	
Mottled duck (*Anas fulvigula*)				H6N5		
Northern Pintail (*Anas acuta*)					H4N8	
Northern shoveler (*Anas clypeata*)				H2N9 H3N8 H4N2 H4N6 H4N8	H4N2 H5N2 H5N3 H6N2 H10N2 H11N9 (2)	H7N2
Teal, blue-winged (*Anas discors*)	H1N1 H3N6 H3N8 (6)	H1N1 (2) H2N8 H3N4 H3N6 H3N8 (9) H4N1 H4N6 (17) H4N8 (6) H6N1 H7N1 H7N1/4 H7N7 (2) H10N7 (5)	H4N6 H4N8	H2N9 H4N2 H4N6 H4N8 H6N1 (3) H6N1/4 H6N5 H6N6 H6N8	H3N6 H5N2 (2) H5N3 (2) H7N4 H7N7 (3) H10N7 H11N9 (3)	H4N8
Teal, green-winged (*Anas crecca*)	H6N2	H10N7		H1N1	H5N2 H7N1/4 H11N9	

[A]Species selected by significance as determined by prevalence, uniqueness to the area, or native, non-migratory species.
[B]Teal are the only species hunted during September on the Texas Gulf coast.

Table 7. Subtypes of avian influenza viruses isolated in the winter (December and January) from select species during three consecutive hunting seasons, September 2006–January 2007 through September 2008–January 2009, Texas mid-Gulf Coast, USA.

Species[A]	December			January		
	2006	2007	2008	2007	2008	2009
Fulvous whistling duck (*Dendrocygna bicolor*)						
Mottled duck x Mallard (*Anas fulvigula x platyrhynchos*)						
Mottled duck (*Anas fulvigula*)						
Northern Pintail (*Anas acuta*)		H10N3/7	H4N6		H10N3	
Northern shoveler (*Anas clypeata*)		H5N2 H6N2 H10N7				
Teal, blue-winged (*Anas discors*)					H10N3 (3)	
Teal, green-winged (*Anas crecca*)	H10N7 H11N3			H7N3	H7N3 H10N3 (2)	

[A]Species selected by significance as determined by prevalence, uniqueness to the area, or native, non-migratory species.

3.4.3. Prevalence by sex, age, species

No statistically significant differences in apparent AIV prevalence were noted between hens and drakes by rRT-PCR or virus isolation during any of the three hunting seasons or all seasons combined (Table 8). Significant differences in AIV apparent prevalence as determined by rRT-PCR and virus isolation were observed between juveniles and adults during the three hunting seasons and all seasons combined (Table 9). However, when data were analyzed based on samples where both sex and age were known, significant differences were noted between adult drakes and hens based on rRT-PCR results during September 2006–January 2007 and between juvenile hens and drakes by virus isolation during September 2008–January 2009 and for all three seasons combined (Table 10).

To determine if there was a species affect on age differences, AIV apparent prevalence by age was assessed for those species where >100 samples from adults and >100 samples from juveniles were tested (i.e., blue-winged teal, green-winged teal, gadwall, and northern shoveler; Tables 11 and 12). When data from all three hunting seasons were combined, significantly more juveniles were positive for AIV than adults based on virus isolation for three of the predominant host species analyzed (blue-winged teal, green-winged teal, and northern shoveler), whereas no significant difference was observed for gadwall (Table 12). However, when rRT-PCR results were analyzed, AIV apparent prevalence was significantly higher only for juvenile blue-winged teal and northern shovelers (Table 12). Based on the multivariate logistic regression model, rRT-PCR results were associated with age and species, but not with sex (Table 4).

Table 8. Apparent prevalence of avian influenza virus in hunter-harvested waterfowl from the Texas Gulf Coast (USA) by sex, year, and test method.

Hunt season	Hen			Drake			p-value (rRT-PCR, VI)
	No.[A]	rRT-PCR[B]	VI[C]	No.	rRT-PCR	VI	
2006-2007	967	7.14% (5.51-8.76)	1.24% (0.54-1.94)	1083	6.10% (4.67-7.53)	1.48% (0.76-2.20)	0.343, 0.645
2007-2008	895	13.30% (11.10-15.50)	4.69% (3.31-6.08)	1059	10.70% (8.89-12.60)	3.77% (2.62-4.92)	0.084, 0.312
2008-2009	400	5.00% (2.86-7.14)	0.25% (0.01-1.38)	303	7.26% (4.34-10.18)	1.32% (0.36-3.35)	0.210, 0.171
Total[C]	2262	9.20% (8.00-10.39)	2.39% (1.76-3.02)	2445	8.26% (7.17-9.35)	2.41% (1.80-3.02)	0.255, 0.956

[A] No.= number tested
[B] rRT-PCR= real-time RT-PCR. Apparent prevalence (95% confidence interval)
[C] VI=virus isolation. Apparent prevalence (95% confidence interval)
[D] Total = all three seasons combined (September 2006–January 2007 through September 2008–January 2009)

Table 9. Comparison of apparent prevalence of avian influenza virus in hunter-harvested waterfowl from the Texas Gulf Coast (USA) by age, year, and test method.

Hunt season	Juvenile			Adult			p-value (rRT-PCR, VI)
	No.[A]	rRT-PCR[B]	VI[C]	No.	rRT-PCR	VI	
2006–2007	518	8.30% (5.92-10.68)	3.28% (1.75-4.82)	1081	5.46% (4.10-6.81)	0.74% (0.23-1.25)	0.029, <0.001
2007–2008	763	13.80% (11.30-16.20)	5.50% (3.89-7.12)	1189	10.51% (8.77-12.20)	3.28% (2.27-4.29)	0.030, 0.022
2008–2009	222	8.56% (4.88-12.24)	1.80% (0.49-4.55)	489	4.70% (2.82-6.58)	0.20% (0.01-1.13)	0.043, 0.035
Total[D]	1504	11.10% (9.52-12.69)	4.06% (3.06-5.06)	2759	1.74% (1.25-2.23)	1.74% (1.25-2.23)	<0.001, <0.001

[A] No.= number tested.
[B] rRT-PCR= real-time RT-PCR. Apparent prevalence (95% confidence interval).
[C] VI=virus isolation. Apparent prevalence (95% confidence interval).
[D] Total = three seasons combined (September 2006–January 2007 through September 2008–January 2009).

Table 10. Apparent prevalence of avian influenza virus in hunter-harvested waterfowl from the Texas Gulf Coast (USA) by age, sex, year, and test method.

Hunting season		2006–07			2007–08			2008–09			Total (2006–09)		
Age	Sex	No. [A]	rRT-PCRI [B]	VI [C]	No.	rRT-PCR	VI	No.	rRT-PCR	VI	No.	rRT-PCR	VI
Adult	Hen	483	34 (7.04%)	5 (1.03%)	468	55 (11.75%)	20 (4.27%)	259	10 (3.86%)	1 (0.39%)	1210	99 (8.18%)	26 (2.15%)
	Drake	580	24 (4.14%)	3 (0.52%)	704	69 (9.80%)	19 (2.70%)	221	13 (5.88%)	0	1505	106 (7.04%)	22 (1.46%)
	Total	1063	58 (5.46%)	8 (0.75%)	1172	124 (10.58%)	39 (3.33%)	480	23 (4.79%)	1 (0.21%)	2715	205 (7.55%)	48 (1.77%)
	p-value		**0.038**	0.331		0.288	0.141		0.301	0.355		0.264	0.177
Juvenile	Hen	250	15 (6.00%)	5 (2.00%)	409	60 (14.67%)	20 (4.89%)	136	10 (7.35%)	0	795	85 (10.69%)	25 (3.14%)
	Drake	254	27 (10.67%)	12 (4.72%)	344	45 (13.08%)	20 (5.81%)	80	9 (11.25%)	4 (5.00%)	678	81 (11.95%)	36 (5.31%)
	Total	504	42 (8.33%)	17 (3.37%)	753	105 (13.94%)	40 (5.31%)	216	19 (8.80%)	4 (1.85%)	1473	166 (11.27%)	61 (4.14%)
	p-value		0.060	0.090		0.531	0.573		0.329	**0.008**		0.448	**0.038**

[A]No.= number tested.
[B] rRT-PCR= real-time RT-PCR. Number positive (apparent prevalence).
[C] VI=virus isolation. Number positive (apparent prevalence).rRT-PCR= real-time RT-PCR. Number positive (apparent prevalence).

Table 11. Multivariate logistic regression model to identify variables associated with a positive rRT-PCR result, 4187 samples included.

Variable	OR[A]	95% CI[B]	p-value of category
Sex			
Drake	1.0 (reference)		
Hen	1.07	0.859-1.32	0.558
Age			
Adult	1.0 (reference)		
Juvenile	1.45	1.17-1.81	**0.001**
Species			
Other species	1.0 (reference)		
Gadwall	0.407	0.120-0.825	**0.013**
Northern shoveler	1.51	0.987-2.32	0.057
Blue-winged teal	2.18	1.52-3.13	**<0.001**
Green-winged teal	1.12	0.742-1.68	0.592

[A] OR= Odds ratio

[B] 95% CI=95% confidence interval

Table 12. Apparent prevalence of avian influenza in blue-winged teal, green-winged teal, gadwall, and northern shoveler, the four most sampled species of hunter-harvested waterfowl from the Texas Gulf Coast by age, sex, and test method, three seasons combined (September 2006–January 2007 through September 2008–January 2009).

	Blue-winged teal			Green-winged teal			Gadwall			Northern shoveler		
	No.[A]	rRT-PCR[B]	VI[C]	No.	rRT-PCR	VI	No.	rRT-PCR	VI	No.	rRT-PCR	VI
Adult	910	11.00% (8.97-13.04)	3.85% (2.60-5.10)	790	6.33% (4.63-8.03)	0.38% (0.08-1.11)	233	2.15% (0.29-4.01)	0.43% (0.01-2.37)	363	7.16% (4.51-9.82)	1.38% (0.18-2.58)
Juvenile	620	15.48% (12.64-18.33)	6.94% (4.94-8.94)	251	7.57% (4.30-10.84)	1.99% (0.26-3.72)	144	3.47% (0.48-6.46)	0	258	12.40% (8.38-16.43)	3.88% (1.52-6.23)
p-value		**0.010**	**0.007**		0.491	**0.011**		0.436	0.431		**0.027**	**0.046**
Hen	772	13.73% (11.30-16.16)	4.40% (2.96-5.85)	548	6.57% (4.50-8.64)	0.91% (0.12-1.71)	221	1.36% (0.28-3.92)	0.45% (0.01-2.50)	373	10.99% (7.82-14.17)	2.68% (1.04-4.32)
Drake	894	11.30% (9.22-13.37)	4.92% (3.50-6.34)	581	7.75% (5.57-9.92)	0.86% (0.11-1.61)	216	3.24% (0.88-5.60)	0	330	7.58% (4.72-10.43)	1.52% (0.20-2.83)
p-value		0.133	0.618		0.444	0.926		0.188	0.322		0.121	0.286

[A] No.= number tested.
[B] rRT-PCR= real-time RT-PCR. Apparent prevalence (95% confidence interval).
[C] VI=virus isolation. Apparent prevalence (95% confidence interval)

Subtype diversity was greatest in blue-winged teal and northern shovelers, followed by green-winged teal (Tables 2–4, 6, and 7). Nine hemagglutinin (H1–7, 10, and 11) and all nine neuraminidase (N1–9) subtypes were identified in blue-winged teal, eight hemagglutinin (H2-7, 10, and 11) and six neuraminidase (N2, 3, and 6–9) subtypes were identified in northern shovelers, whereas six hemagglutinin (H1, 5–7, 10, and 11) and six neuramindase (N1–4, 7, and 9) subtypes were identified in green-winged teal.

3.5. Discussion

The Texas Gulf Coast provides winter habitat for approximately two to three million ducks and over a million geese (DU, 2008). In this region migratory waterfowl intermingle with resident wild species such as the mottled duck, and are in close contact with poultry operations and humans, primarily hunters (Bellrose, 1978; Miller, 2007). Recently, we reported prevalence for the first multiyear study of AIV covering waterfowl wintering grounds along the Texas Gulf Coast (Ferro et al., 2010), a previously understudied area. Unlike previous studies, we found little to no variation in apparent AIV prevalence by month within wintering seasons (September–January) with the exception of rRT-PCR during 2008–2009 and virus isolation during 2006–2007 and 2007–2008 (Ferro et al., 2010). Additionally, little variation in AIV prevalence as determined by rRT-PCR or virus isolation was noted among the four consecutive hunting seasons studied (September 2005–January 2006 through September 2008–January 2009) with the exception of the 2007–2008 season where overall AIV prevalence was higher than the other three seasons by both rRT-PCR and virus isolation (Ferro et al., 2010). Detection of AIV at low levels throughout the wintering season

supports the contention that AIV can persist in wild bird populations through continuous circulation in a proportion of the population (Webster et al., 1992). The low rate of virus isolation observed in the current study (29.89% of rRT-PCR positive samples) is consistent with other studies and is not surprising (Dusek et al., 2009; Munster et al., 2007; Siembieda et al., 2010). Real-time RT-PCR is considered more sensitive than virus isolation enabling the detection of genome fragments as well as detection of viruses that do not grow in embryonated chicken eggs. Also, consistent with other surveillance studies, no differences were noted in AIV prevalence based on sex, and AIV was more prevalent in juvenile birds than adults ((Krauss et al., 2004; Munster et al., 2007; Webster et al., 1992). The significantly higher AIV prevalence in juveniles as compared to adults supports the assumption that immunologically immature (juvenile) birds are more susceptible to AIV as compared to mature (adult) waterfowl (Stallknecht and Brown, 2007; Stallknecht and Shane, 1988).

The most commonly identified hemagglutinin and neuraminidase combinations during the first sampling season (September 2006–January 2007) were H3N8 and H6N1 with H3N8 remaining during the second season (September 2007–January 2008), but not detected during the third (September 2008–January 2009). This is noteworthy because H3N8 had previously not been reported in waterfowl from the Texas Gulf Coast even though this subtype is commonly isolated elsewhere in North America (Ferro et al., 2008; Hanson et al., 2005; Krauss et al., 2004). During the 2007–2008 season, H4N6 and H10N7, which have been reported on the Gulf Coast (Ferro et al., 2008; Stallknecht et al., 1990), were the predominant subtype combinations; H4N6 also was detected

during September 2008–January 2009. The annual variations in AIV subtype prevalence observed in this study add support to the need for continued annual surveillance in domestic as well as migratory avian species. This is particularly true in areas of high poultry and waterfowl density such as the Texas Gulf Coast (Miller, 2007).

Outbreaks of H5 AIV were documented previously in Texas. In 1993, an outbreak of H5N2 occurred in emus, in 2002 H5N3 was detected in chickens, and in 2004 HPAIV H5N2 was reported in a commercial poultry operation (Lee et al., 2004; Lee et al., 2005; Pelzel et al., 2006). We isolated H5N2 and H5N3 viruses from apparently healthy free-roaming waterfowl only during the 2007–2008 hunting season. Although no data are available concerning subtypes circulating in waterfowl on the Texas coast prior to the three outbreaks listed above, our data document the presence of these subtypes in migratory waterfowl in close proximity to commercial poultry operations (Miller, 2007). Molecular characterization of the H5N2 and H5N3 viruses we isolated should help clarify the relationship between these viruses and those isolated from commercial species.

Our isolation of AIVs from resident (non-migratory) mottled ducks and mottled duck/mallard hybrids suggests AIV transmission on the wintering ground and is consistent with previous reports (Stallknecht et al., 1990). Mallards interbreed with mottled ducks and are sister species phylogenetically (Omland, 1994). Prior to the isolation of H6 AIVs from a mottled duck/mallard hybrid in November 2006, and a mottled duck in November 2007, we isolated H6 subtypes from migratory teals and northern shovelers (September and November 2006 and 2007). Additional support for

AIV transmission on wintering grounds included the isolation of an H6 virus from a fulvous whistling duck, a species which breeds on the Texas-Louisiana coast and leaves during late summer to winter farther south in Mexico, with nearly all individuals gone by late January (Bellrose, 1978). Although circulation of AIVs within fulvous whistling ducks, mottled ducks, and mottled duck hybrids throughout the year cannot be ruled out, this seems unlikely. Hanson et al. (2005 were unable to isolate AIVs from mottled ducks collected on the Texas Gulf Coast during August (Hanson et al., 2005); additionally, we failed to detect AIV by rRT-PCR in samples collected during June–August 2007 (no.=155, S. Rollo unpublished data), suggesting that these viruses are not readily circulating in these resident populations during summer. Genetic characterization of these H6 isolates will help determine whether these isolates are related and help clarify the role of waterfowl wintering grounds on transmission and perpetuation of AIVs in nature. Further studies focused on AIV prevalence and immune responses to AIV in these resident populations are also needed to better understand the maintenance and transmission of AIV in the wintering grounds.

Prior to singling out a particular species upon which to focus surveillance efforts, one must consider the technique used for subject selection (hunter-harvest vs. live-capture) as well as the area under study (i.e. breeding grounds vs. wintering grounds; fresh water vs. salt water, etc.) and which populations are prevalent within the study areas. Mallards have become a primary species of interest not only because of their susceptibility to H5 and H7 subtypes but also because of their abundance and relative ease of capture (Bellrose, 1978; Dusek et al., 2009; Jourdain et al., 2010; Munster et al.,

2007; Olsen et al., 2006; Wallensten et al., 2007). During our study, few mallard samples were collected because most mallards in Texas winter in the playa lakes and sorghum fields of the Texas panhandle with few (<4%) wintering along the Gulf Coast (Bellrose, 1978). Further, many duck hunters along the Texas Mid-Coast typically choose other species to harvest as they view mallards as essentially free-roaming captive-reared ducks (Ferro, personal observation). Our data indicate that mallards, while appropriate focal species for AIV monitoring in some portions of North America, are not as suitable as blue-winged teal or northern shoveler in other regions such as the Texas Mid-Coast (Ferro et al., 2008; Hanson et al., 2005; Siembieda et al., 2010; Stallknecht et al., 1990). Interestingly, in many studies where mallards emerged as a high prevalence species for AIV infection, they were captured live for testing and dominated the samples (Dusek et al., 2009; Munster et al., 2007). The few studies where other species were more frequently sampled and tested positive for AIV were conducted on hunter-harvested waterfowl (Ferro et al., 2008; Siembieda et al., 2010; Stallknecht et al., 1990).

Our study supports the consensus that dabbling ducks are more likely to be positive for AIV than diving ducks; however, as others have documented, not all dabbling ducks are equally likely to be AIV positive. We found blue-winged teal to be the highest prevalence species followed by northern shoveler and green-winged teal. Gadwalls, also a dabbling duck from which we collected substantial numbers of samples, were the least likely to test positive for AIV. Blue-winged teal are generally the first ducks to fly south in the fall, first arriving on wintering grounds beginning in

September, and the last ones to pass through Texas in late February through March on their return north (Bellrose, 1978). They also make exceedingly long flights compared to other dabbling ducks between feeding and resting areas during migrations (Bellrose, 1978). On the other hand, gadwalls are short-distance migrants and migrate later, generally beginning their southward migration in early September and their return north starting in February (Bellrose, 1978). The physiologic demands of long-distance migration can suppress the immune system (Weber and Stilianakis, 2007); thus it is possible that blue-winged teal may be more susceptible to infection than some other dabbling ducks due to their long distance migration. More extensive studies are needed incorporating more ecological factors such as food resources, body mass, and immune status in order to more fully understand how AIV persist in nature and why higher prevalence of AIV is observed in particular species.

Although our samples were not collected probabilistically (i.e., the samples reflect hunter's choices as well as the relative abundance of each species), by targeting hunter-harvested waterfowl, we were able to estimate the prevalence of various AIV subtypes carried by waterfowl in the Gulf Coast of Texas to which humans are most likely to be exposed (Siembieda et al., 2008). As recent reports have shown, antibodies to several AIV subtypes were detected in hunters and wildlife professionals that are not commonly found in other people (primarily H5, H6, H7, and H11), demonstrating exposure to AIVs circulating in waterfowl populations (Gill et al., 2006; Myers et al., 2007; Siembieda et al., 2008). Thus continued monitoring of AIV in waterfowl and

humans exposed to these species should provide important information regarding the prevalence and significance of wild animal-to-human transmission.

In summary, AIV surveillance studies over time in the same region are critical, particularly in understudied areas. Although studies in low AIV prevalence areas are inconvenient due to the large sample sizes required in order to isolate significant numbers of AIVs, such surveys are critical to better understand the ecology of influenza viruses and their impact on human health. Our data contribute temporal information on AIV prevalence and subtype diversity for a historically understudied area of North America, the waterfowl wintering grounds of the Texas Gulf Coast.

CHAPTER IV

COMPARISON OF REAL-TIME RT-PCR AND VIRUS ISOLATION FOR ESTIMATING PREVALENCE OF AVIAN INFLUENZA IN HUNTER-HARVESTED WILD BIRDS AT WATERFOWL WINTERING GROUNDS, TEXAS MID-GULF COAST (2005–2006 THROUGH 2008–2009)*

4.1. Overview

Historically, virus isolation has been the method of choice for conducting surveillance for avian influenza virus in avian species. More recently, the primary screening method has become real-time reverse-transcription polymerase chain reaction (rRT-PCR). We wanted to determine how these two testing methods (virus isolation and rRT-PCR) affected AIV prevalence estimation, particularly in an understudied, low prevalence region—the waterfowl wintering grounds along the Texas mid-Gulf Coast. Cloacal swabs were collected from hunter-harvested waterfowl and other wetland-associated gamebirds during four consecutive hunting seasons (2005–2006 through 2008–2009). Overall prevalence by rRT-PCR (5.9, 6.5, 11.2, and 5.5%) was approximately an order of magnitude higher than prevalence by virus isolation (0.5, 1.3, 3.9, and 0.7%) for the four hunting seasons, respectively. Apparent AIV prevalence by

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virus isolation conducted only on rRT-PCR positive samples resulted in estimates nearly identical in magnitude to those derived from parallel testing (0.5 v. 0.6%, 1.3 v. 1.7%, and 3.9 v. 4.0% for 2005–2006, 2006–2007, and 2007–2008, respectively). Unlike most reports of seasonal variation in AIV prevalence, we documented differences in prevalence estimates among months using rRT-PCR only during 2008–09 and by virus isolation only during 2006–2007 and 2007–2008. Our data indicate screening samples by rRT-PCR followed by virus isolation only on rRT-PCR positive samples, provides a reasonable means to generate prevalence estimates close to the true prevalence as determined by virus isolation. We also confirmed the low prevalence of AIV in waterfowl wintering grounds along the Texas mid-Gulf Coast and demonstrated little variation in prevalence among months during the four hunting seasons when we collected samples.

4.2. Introduction

Wild waterfowl are considered the natural reservoir of type A influenza viruses (Webster et al., 1992). The migratory nature of many waterfowl species and the persistence of influenza virus infection in these populations present a potential vehicle for dissemination of influenza viruses globally. Understanding the migratory patterns of different waterfowl populations, as well as identifying influenza virus subtypes within these populations, is critical to our understanding of how influenza viruses persist in nature and evolve over time. As concerns over the spread of highly pathogenic avian influenza (HPAI) H5N1 viruses have increased, surveillance programs have been implemented worldwide. The primary method utilized internationally for screening

samples for avian influenza virus (AIV) is real-time reverse-transcription polymerase chain reaction (rRT-PCR) for the matrix gene, a highly conserved gene in type A influenza viruses (Agriculture, 2006; Cattoli et al., 2007; Pasick, 2008). Virus isolation also has been used in some cases, primarily for confirmation in the event of positive rRT-PCR results. Additionally, within many of these surveillance programs, any sample positive for type A influenza virus is further screened by rRT-PCR specific for H5 and H7 subtypes, the two subtypes most commonly associated with losses in poultry (Alexander, 2000) and classified as notifiable AIV by the World Organization for Animal Health (OIE) (OIE, 2009).

Prior to the implementation of surveillance programs focused on identifying HPAI H5N1, most studies reporting prevalence of influenza viruses in North America were conducted during the late summer to early fall when pre-migration staging occurs, and therefore were concentrated in Alaska, Canada, the upper Midwestern USA, and the Northeastern USA (Krauss et al., 2004; Webster et al., 1992). Few studies involved waterfowl on their wintering grounds or non-migratory waterfowl during winter; this is particularly true along the Gulf Coast, where such studies were limited to just a few waterfowl species (Ferro et al., 2008; Hanson et al., 2005; Stallknecht et al., 1990). Additionally, in these studies, virus isolation was the primary method for screening samples and identifying AIV, hence prevalence estimates were based on these results. The objectives of this study were: i) to compare prevalence estimates obtained using rRT-PCR to estimates obtained using virus isolation; ii) to determine how prevalence estimates based on virus isolation conducted only on rRT-PCR positive samples

compares to estimates obtained from paired samples tested in parallel (rRT-PCR and virus isolation); iii) and to estimate prevalence of AIV on waterfowl wintering grounds along the Texas mid-Gulf Coast, a low prevalence region, during four consecutive years.

4.3. Materials and methods

4.3.1. Sample collection

Hunter-harvested waterfowl (Bellrose, 1978) and other wetland-associated gamebirds (Tacha and Braun, 1994) were sampled during four consecutive hunting seasons (2005–2006 through 2008–2009) at four state wildlife management areas (WMA) along the Gulf Coast of Texas: Justin Hurst WMA in Brazoria County, Mad Island WMA in Matagorda County, Guadalupe Delta WMA and Matagorda Island in Calhoun County. Dates of collection were similar across all seasons and included days when hunters were most likely to be in the field in order to capitalize on harvest. Species collected and areas sampled reflect hunters' choices and personnel available to collect swabs on sampling days, as well as the relative abundance of hunted species. Data from all four WMAs were combined for analysis.

Cloacal swabs were collected within 6 hr of waterfowl harvest using sterile Dacron swabs (Fisher Scientific, Houston TX, USA) and placed in 3 ml tryptose phosphate broth (Becton Dickinson NJ, USA) supplemented with antibiotics [penicillin G ($2x10^3$ U/ml), streptomycin (200 µg/ml), gentamicin (250 µg/ml), and amphotericin B ($2x10^3$ U/ml) (Sigma, St. Louis MO, USA)]. Samples were transported from the field on wet ice (<10 hours collection and transport time) and stored at -80ºC until processed.

4.3.2. Real-time RT-PCR

Cloacal swab samples were thawed, vortexed, and centrifuged at 1,500 × g for 10 min and 100 µl of supernatant was dispensed into 96-well plates for RNA isolation. The remainder of the samples and 96-well plates were frozen at -80ºC until processed for virus isolation (sample tube) or rRT-PCR (96-well plates). For rRT-PCR, 96-well plates were thawed and RNA was extracted using a magnetic particle processor in a 96-well format (KingFisher 96, ThermoScientific, USA) and the MagMax™-96 AI/ND RNA Isolation Kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Extracted RNA was transferred to nuclease-free 96-well plates for immediate use. The rRT-PCR assay was performed using the AgPath-ID™ AIV-M Reagent Kit (Ambion, Inc., Austin, TX), a one-step rRT-PCR for the detection of AIV matrix gene RNA, as per manufacturer's instructions and an ABI 7900HT (Applied Biosystems, Inc., USA) thermocycler in a 384-well format with a 15 µl final reaction volume using 5 µl extracted RNA (Ferro et al., 2009). Primers and probe for the AIV matrix gene were those previously described (Spackman et al., 2002).

4.3.3. Virus isolation

Cloacal swab samples were thawed and 0.2 ml was inoculated via the allantoic route into four 9–10 day-old embryonated chicken eggs. Eggs were incubated at 37ºC for five days and examined daily for deaths. Any eggs dying within 24 hr post-inoculation were considered non-specific and discarded. Amnio-allantoic fluid (AAF) was collected from surviving embryos and any embryos dying more than 24 hr post-inoculation. All fluids were tested for hemagglutination (HA) activity and all HA positive fluids were

further analyzed for the presence of influenza virus using a rapid immuno-migration assay designed to detect type A influenza (FluDetect®, Synbiotics Inc., USA). Fluids testing positive for influenza virus were sent to the National Veterinary Services Laboratory (NVSL), in Ames, IA, USA, for confirmation.

4.3.4. Data analysis

All samples (6,823) in this study were tested using rRT-PCR. A number of samples (4,820) were tested in parallel using both rRT-PCR and virus isolation. In this case, apparent prevalence was calculated by dividing the number of positive samples by method (i.e., virus isolation or rRT-PCR) by the total number of samples processed. For some samples (541), virus isolation was attempted only after a positive rRT-PCR was obtained. In this case, apparent prevalence by virus isolation was calculated by dividing the number of virus isolation positive samples by the total number of samples collected and tested using rRT-PCR.

Screening samples with rRT-PCR and attempting virus isolation only on rRT-PCR-positive samples has become a standard technique for AIV surveillance (Cattoli et al., 2007; Dusek et al., 2009; Munster et al., 2009; Munster et al., 2007). Since differences in prevalence estimates obtained using this approach versus those obtained if virus isolation is attempted on all samples is not nearly as relevant as whether such differences are sufficiently large to alter how we interpret these results, 95% confidence intervals (CI) around all apparent prevalence estimates were calculated. This approach allowed to simultaneously evaluate whether differences occurred at the $\alpha<0.05$ level and, more importantly, evaluate the magnitude of these differences. Specifically, we use this

approach: i) to compare and contrast apparent AIV prevalence in water birds collected along the Texas mid-coast when samples were subjected to both rRT-PCR and virus isolation (i.e., virus isolation and rRT-PCR regardless of a result by the other test method); ii) to determine how these results compare to those obtained when viral isolation was only attempted on rRT-PCR positive samples; iii) and to determine how apparent AIV prevalence as estimated by rRT-PCR and virus isolation conducted on rRT-PCR positive samples varied among sampling months and years.

4.4. Results and discussion

We collected 6,822 swabs over four years (2005–2006: 1,460; 2006–2007: 2,171; 2007–2008: 2,423; and 2008–2009: 768) from 30 different potential host species, including a variety of waterfowl, nine other wetland-associated gamebird species, and two additional avian species from five orders (Table 13). Most samples (88.3%) were from dabbling ducks (genus *Anas*), while diving ducks (genus *Aythya*) accounted for 5.0%, and geese (genera *Anser, Chen* and *Branta*) 3.0% of the samples tested, with waterfowl (Anatidae) comprising 98.7% of samples. Of the waterfowl (Anatidae) sampled, 1.8% were from non-migratory dabbling ducks (genus *Anas*). The remaining samples were collected from six potential host species, and in three cases only one sample was tested per species (Table 13). Although our samples reflect hunter's choices as well as the relative abundance of each species, hunter-harvested waterfowl provide an economical method by which to conduct waterfowl surveillance and targets potential host species humans are most likely to come into contact with.

Table 13. Wild waterfowl[A] and other wetland-associated gamebirds[B] sampled on the Texas Gulf Coast during the 2005–2006 through 2008–2009 hunting seasons.

Order	Family	Genus	Number of species	Number of samples
Anseriformes	Anatidae [A]	*Dendrocygna*	2	57
		Anser	2	32
		Chen	1	169
		Branta	1	4
		Aix	1	4
		Anas	9	6025
		Aythya	4	344
		Bucephala	2	4
		Laphodytes	1	22
		Oxyura	1	76
Podicipediformes	Podicipedidae	*Tachybaptus*[C]	1	1
Gruiformes	Rallidae	*Gallinula*[B]	1	1
		Fulica[B]	1	65
	Gruidae	*Grus*[B]	1	14
Charadriiformes	Scolopacidae	*Gallinago*[B]	1	4
Columbiformes	Columbidae	*Columbina*[D]	1	1
Total			30	6823

[A] The term *waterfowl* refers to the Family Anatidae (ducks, geese, and swans).
[B] Other wetland-associated gamebirds sampled included a common moorhen (*Gallinula chloropus*), American coots (*Fulica americana*), sandhill cranes (*Grus canadensis*), and Wilson's snipe (*Gallinago delicata*).
[C] The least grebe (*Tachybaptus dominicus*) is a non-game wetland bird made available for sampling at our sampling location.
[D] The common ground dove (*Columbina passerina*) is an upland gamebird collected by a hunter at our sampling location.

Both rRT-PCR and virus isolation were conducted in parallel on 4,820 samples collected during three hunting seasons (2005–2006 through 2007–2008). Apparent prevalence estimates for consecutive hunting seasons estimated by rRT-PCR (3.6, 6.7, and 11.2%, respectively) were significantly and substantially higher than those estimated by virus isolation (0.6, 1.7, and 4.0%, respectively) (Table 14). Our prevalence estimates as determined by virus isolation (Table 14) are consistent with those previously described in studies of the Gulf Coast waterfowl wintering grounds, which ranged from 0.5 to 10.0% (Ferro et al., 2008; Hanson et al., 2005; Stallknecht et al., 1990). No other reports of apparent AIV prevalence based solely on rRT-PCR are available for water bird wintering areas along the Gulf Coast, therefore no comparison to historical estimates can be made.

Apparent AIV prevalence by virus isolation conducted only on rRT-PCR positive samples (Table 15) resulted in estimates nearly identical in magnitude to those derived from parallel testing (Table 2) (0.5 v. 0.6, 1.3 v. 1.7, and 3.9 v. 4.0 for 2005–06, 2006–2007, and 2007–2008, respectively). Moreover, each prevalence estimate using one approach fell near the center of the 95% CI for the analogous estimate derived using the alternative approach (Tables 14–15). By using rRT-PCR as a screening tool, however, two isolates listed in Table 14 would not have been identified.

Prior to the widespread usage of molecular screening tools for AIVs, prevalence estimates were based solely on virus isolation. Recent increases in avian influenza surveillance and the high cost of virus isolation have lead diagnosticians and researchers to utilize rRT-PCR as a screening method, followed by virus isolation only on rRT-PCR-

positive samples (Cattoli et al., 2007; Dusek et al., 2009; Munster et al., 2009; Zohari et al., 2008). Our data support using rRT-PCR as a screening tool and performing virus isolation only on rRT-PCR positive samples. In order to provide accurate prevalence estimates, this approach requires that the number of virus isolation positive samples be divided by the total number of samples tested by rRT-PCR and not the number of samples tested by virus isolation.

Differences observed in apparent prevalence estimates between virus isolation and rRT-PCR are not surprising. The rRT-PCR is more sensitive than virus isolation, primarily because it can detect genome fragments and does not require the presence of intact infectious particles. Munster et al. reported the successful isolation of AIV in 33.5% of rRT-PCR positive samples (Munster et al., 2007). In this study, we successfully isolated AIV in 25.1% of rRT-PCR positive samples (Table 15; 136 of 541 samples). These differences could be attributed to the absence of cryo-protectant, such as bovine serum albumin or glycerol, in our samples prior to freezing. Studies using paired samples frozen with or without a cryo-protectant could clarify whether a cryo-protectant would influence virus recovery.

Table 14. Apparent prevalence of avian influenza virus in cloacal swabs collected from hunter-harvested waterfowl from the Texas Gulf Coast during three consecutive hunting seasons (2005–2006 through 2007–2008) in paired samples tested by both rRT-PCR and virus isolation regardless of rRT-PCR result.[A]

		rRT-PCR			Virus isolation		
Hunting season	Number sampled	Number positive	Apparent prevalence (%)	95% CI	Number positive	Apparent prevalence (%)	95% CI
2005–06	776	28	3.6	2.30–4.92	5	0.6	0.08–1.21
2006–07	1620	108	6.7	5.45–7.88	29	1.7	1.14–2.44
2007–08	2424	272	11.2	9.96–12.48	97[B]	4.0	3.22–4.78
Total	4820	408	8.5	7.68–9.25	131[B]	2.7	2.26–3.18

[A] Only samples tested by both methods regardless of results of the other test method were included in this analysis.
[B] Includes two samples negative by rRT-PCR, yet positive by virus isolation.

Table 15. Apparent prevalence of avian influenza virus in cloacal swabs collected from hunter-harvested waterfowl from the Texas Gulf Coast during four consecutive hunting seasons (2005–2006 through 2008–2009) as determined by rRT-PCR and virus isolation following a positive rRT-PCR result.

		rRT-PCR			Virus isolation following rRT-PCR positive		
Hunting season	Number sampled	Number positive	Apparent prevalence (%)	95% CI	Number positive	Apparent prevalence (%)[A]	95% CI
2005–06	1460	86	5.9	4.68–7.10	7	0.5	0.13–0.83
2006–07	2171	141	6.5	5.46–7.53	29	1.3	0.85–1.82
2007–08	2424	272	11.2	9.96–12.48	95[B]	3.9	3.14–4.70
2008–09	768	42	5.5	3.86–7.08	5	0.7	0.08–1.22
Total	6823	541	7.9	7.29–8.57	136[B]	2.0	1.66–2.32

[A] Apparent prevalence calculated using total sampled by rRT-PCR as denominator.
[B] Excludes two samples negative by rRT-PCR, yet positive by virus isolation.

Unlike previous reports of seasonal variation in AIV prevalence (Halvorson et al., 1985; Munster et al., 2009; Stallknecht et al., 1990), we documented differences in prevalence estimates among months using rRT-PCR only during 2008–2009 and by virus isolation only during 2006–2007 and 2007–2008 (Fig. 3). Although significant differences in total prevalence estimates were observed between rRT-PCR and virus isolation during most months, confidence intervals overlapped four times (January and September 2006, November and December 2008; Fig. 3). Additional surveys including all months of the year as well as other wintering grounds would provide valuable data regarding AIV prevalence and persistence in these understudied areas.

As with any study, the most important aspect to consider when deciding which detection method to use (i.e. rRT-PCR and/or virus isolation) is the goal of sampling (i.e. process more samples or obtain all possible isolates). Over the four-year course of our study, we performed virus isolation on 4,953 samples (4,820 samples tested in parallel and an additional 133 following a positive rRT-PCR result) and rRT-PCR on 6,824 samples, respectively, resulting in 138 AIV isolates, of which two were obtained from rRT-PCR negative samples. Thus, if the focus of the surveillance program is processing as many samples as possible, screening by rRT-PCR, and then performing virus isolation only on rRT-PCR positive samples, provides a reasonable means to process a large number of samples and generate prevalence estimates quite similar to the true prevalence as determined by virus isolation conducted on all samples (Tables 14–15). Additionally, our data confirm the low prevalence of AIV in waterfowl wintering grounds along the

Texas mid-Gulf Coast and demonstrate little variation in prevalence among months during the four hunting seasons when we collected samples.

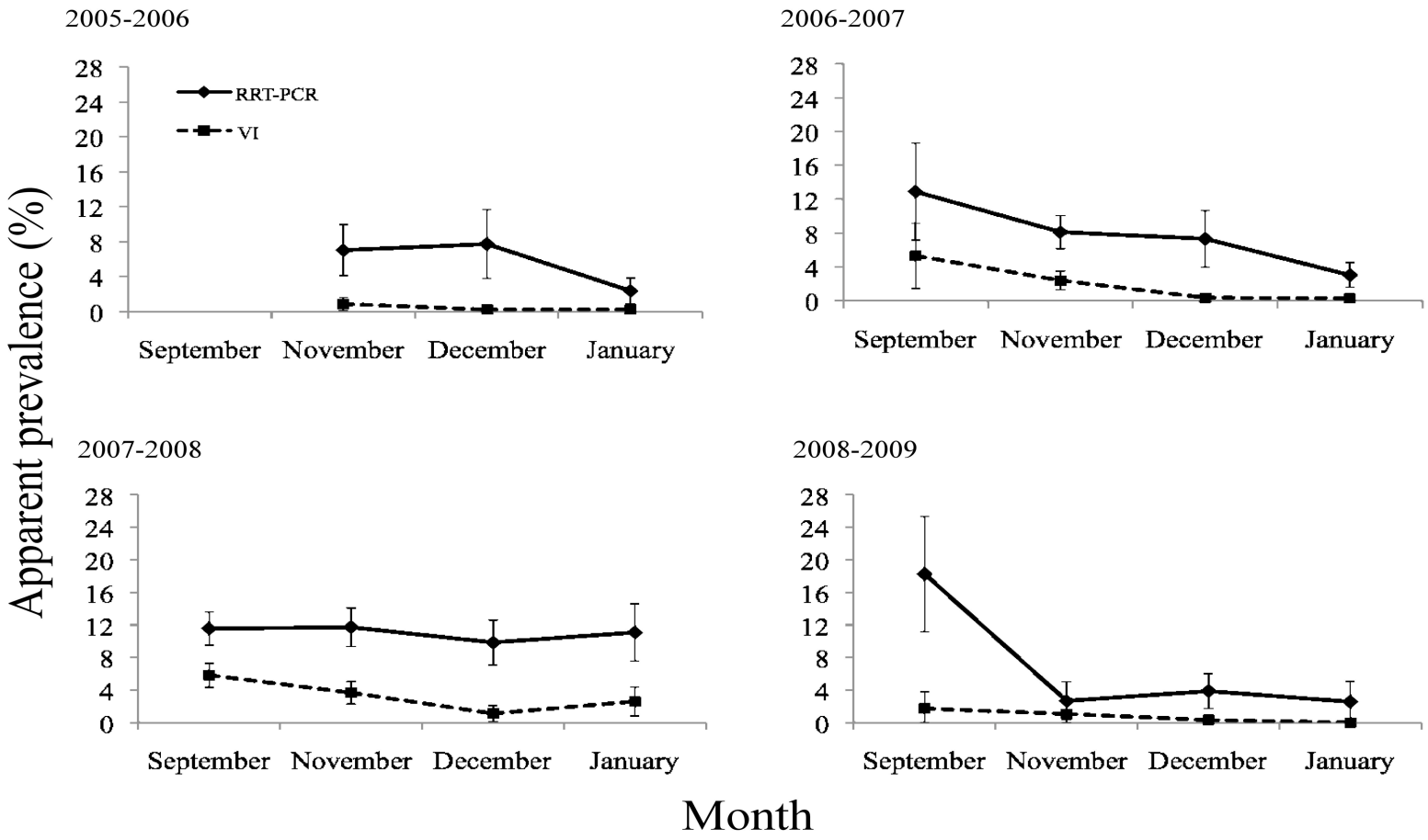

Fig. 3. Apparent prevalence of avian influenza in hunter-harvested waterfowl from the Texas mid–Gulf Coast as determined by rRT-PCR and virus isolation following a positive rRT-PCR result by hunting season (2005–2006 through 2008–2009), month, and test method. The bars represent 95% confidence intervals.

CHAPTER V

MOLECULAR ANALYSIS OF SELECT AVIAN INFLUENZA VIRUS ISOLATES FROM TEXAS WATERFOWL

5.1. Introduction

Influenza viruses are members of the *Orthomyxoviridae* family. This family is characterized by a segmented, single-stranded, negative sense RNA genome. Influenza A viruses are classified into subtypes based on two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) (Palese and Shaw, 2007). Currently, there are 16 different HA subtypes (H1–16) and nine different NA subtypes (N1–9), which can be found in up to 144 different combinations. The HA is the major surface protein of influenza A viruses and is responsible for attachment of the virion to a susceptible cell. Pathogenicity is most often associated with the HA protein due to its binding activity to different surface receptors and cleavability by host cell proteases, which is required for activation (Steinhauer, 1999). The second major glycoprotein of influenza A virus is the NA. The NA, due to its enzymatic activity, functions to remove sialic acid and is primarily responsible for the release of progeny virus from an infected cell (Palese and Shaw, 2007).

Due to the segmented nature of the influenza genome and the lack of proofreading of the viral polymerase, influenza viruses are able to change genetically fairly readily. The two methods by which new strains develop are through “genetic drift” and “genetic shift”. Genetic drift is a consequence of point mutations due to the lack of proofreading of the viral RNA dependent RNA polymerase. Genetic shift occurs when a

cell becomes infected with at least two different influenza viruses resulting in the exchange of segments (gene reassortment) between the two viruses. When genetic changes occur involving the HA or NA genes, the changes are commonly referred to as "antigenic drift" and "antigenic shift". Antigenic drift is the method by which epidemics typically arise, whereas antigenic shifts are the method by which pandemics typically arise. Antigenic drift also renders the virus less susceptible to immediate neutralization by the immune system in individuals with previous exposure via either vaccination or infection (McHardy and Adams, 2009).

Depending on their virulence in poultry, AIVs are further classified as low pathogenic (LP) or highly pathogenic (HP) viruses (Capua and Alexander, 2004). Whereas LPAIV strains can cause asymptomatic to mild respiratory and enteric tract infections, HPAIV strains cause clinical illness and systemic disease and may cause mortality as high as 100%. Until now, only viruses from the H5 and H7 subtypes have been classified as HPAIV, but not all H5 and H7 viruses are HPAIV (Alexander, 2000). Wild aquatic water birds are considered the natural reservoir for all type A influenza viruses due to the fact that all hemagglutinin and neuraminidase combinations have been identified in these species. HPAIV do not occur naturally in wild waterfowl and only after passage through domestic poultry do these viruses undergo genetic changes that result in HPAIV (Alexander, 2000). Prior to the emergence of HPAI H5N1, with the exception of A/tern/South Africa/1/61 (Becker, 1966), no clinical signs of disease or mortalities due to natural infections with AIV were reported in wild or domestic ducks (Alexander, 2000). Recently, however, one report identified an H5N2 virus circulating in

healthy wild waterfowl with the molecular signature of a highly pathogenic virus (Gaidet et al., 2008). Since the emergence of HPAIV H5N1, these viruses have been transmitted from poultry to wild waterfowl and in outbreaks across Asia, the Middle East, Europe, and Africa, wild birds now are suspected of playing a role as long-distance vectors of AIVs (Keawcharoen et al., 2008). The migratory nature of water birds and the overlapping of the different flyways provide an environment for the exchange of viruses that could spread to new regions; however, the role of wild birds in the spread of HPAI remains to be determined. Until recently, studies analyzing the evolutionary aspects of AIV in reservoir species have rarely been undertaken and most have focused on comparison of wild bird influenza virus sequences to isolates from outbreaks in poultry (Chin et al., 2002; Hoffmann et al., 2000; Webby et al., 2002). Limited numbers AIVs from reservoir species in the Central Flyway of North America have been identified, so few sequences are available for comparison to other regions.

The objectives of this study were to sequence the HA (H6, H5, and H7) and NA (N1, 2, 3, and 4) genes from type A influenza wild bird isolates from Texas Gulf Coast hunter-harvested waterfowl as part of a multiyear surveillance study (Chapter II and III) and compare these new sequences to other publicly available sequences from other avian isolates, primarily waterfowl. Phylogenetic analyses were used to determine the relationships between nucleotide sequences of the HA and NA open reading frame (ORF) from the new Texas isolates and those publicly available in the GenBank (http://ncbi.nlm.nih.gov) and BioHealth (Squires et al., 2008) databases.

5.2. Materials and methods

5.2.1. Avian influenza virus (AIV) isolates

AIV isolates from both migratory and non-migratory water birds, which were isolated from hunter-harvested waterfowl as part of an ongoing surveillance project were analyzed herein (Chapters II and III). Eight H5, 16 H6, 13 H7, nine N1, nine N2, six N3, and three N4 genes were included in this study (Table 16). AIVs were isolated using standard methods as previously described (Chapters II and III). Allantoic fluid from the infected eggs was utilized as the virus source. Isolates were passaged no more than two times in embryonated chicken eggs prior to sequencing.

5.2.2. Sample processing and sequencing

Briefly, RNA was extracted from allantoic fluid using the MagMax™ AI/ND Viral RNA Isolation Kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. First-strand cDNA was synthesized by adding 2 µl (10 µM) Uni12 primer (Hoffmann et al., 2001) and 3 µl of RNA to 7 µl of nuclease free water to give a 12 µl reaction volume. The reaction was heated to 70°C for 5 min. and then transferred immediately to ice. RNA-primer mix was then added to 2 µl 10x RT buffer, 4 µl dNTP mix (10 µM total, 2.5 µM each; Epicentre Technologies Corp, Madison, WI), 1 µl RNasin (40 U/µl; Invitrogen, Carlsbad, CA), and 1 µl (100 U/µl) M-MLV polymerase (Ambion, Austin, TX) making a final volume of 20 µl. The reaction conditions were 25°C for 5 min, 42°C for 60 min, 92°C for 10 min, and a final hold of 4°C. Amplification of the HA and NA genes was accomplished by generating overlapping fragments of approximately 500–600 bp with in-house designed primers (H6 and some

H5) or published primers (H7, H5, N1, N2, N3, N4; http://gsc.jcvi.org/projects/msc/influenza/infl_a_virus/primers.shtml) (Table 17). Primers were designed with M13 forward and reverse specific sequences at the 5' end. The PCR conditions were 95°C for 4 minutes, 35 cycles of 95°C for 30 sec, 50–60°C for 30 sec, 72°C for 2 minutes, a final extension of 72°C for 10 minutes, and a final hold of 10°C. The PCR products were visualized on a 1% agarose gel containing ethidium bromide and PCR products showing a single band were purified using a PCR clean-up kit (PureLink PCR purification kit, Invitrogen). All PCR products producing multiple bands were gel purified using a gel purification kit according to manufacturer's instructions (QIAEX II, QIAGEN Sciences, MD) with the exception that all incubations were conducted at room temperature. DNA was quantified (Nanodrop, Thermo Electron Corp., Wilmington, DE) and the recommended concentration of product was used as template in sequencing reactions according to manufacturer's instructions (Big Dye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). The primary sequencing primers were M13 forward (5'-TGTAAAACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGACC-3'), and other primers, based on the sequences obtained, were designed to fill gaps (Table 17). Following the sequencing reaction, excess terminators were removed (Spin-50, USA Scientific, Ocala, FL) and ready-to-run sequencing reactions were submitted to the DNA Core Facility at Texas A&M University for sequencing. Sequences were analyzed, contigs assembled, and consensus sequences determined using Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI).

Table 16. List of H5, H6 and H7 AIV isolates collected from hunter-harvested waterfowl along the Texas mid-Gulf Coast during four consecutive hunting seasons (November 2005–January 2006 through September 2008–January 2009) for which the HA and/or NA gene sequences were determined and examined in this study.

Isolate	Date of collection	Gene segment[A]	
		HA	NA
A/blue-winged teal/TX/1087/2007(H5N2)	November 3, 2007	X	X
A/green-winged teal/TX/1207/2007(H5N2)	November 3, 2007	X	X
A/northern shoveler/TX/1210/2007(H5N3)	November 3, 2007	X	X
A/blue-winged teal/TX/1402/2007(H5N2)	November 3, 2007	X	X
A/blue-winged teal/TX/1459/2007(H5N3)	November 3, 2007	X	X
A/blue-winged teal/TX/1473/2007(H5N3)	November 3, 2007	X	X
A/northern shoveler/TX/1578/2007(H5N2)	November 10, 2007	X	X
A/northern shoveler/TX/2003/2007(H5N2)	December 8, 2007	X	X
A/green-winged teal/TX/G39/2005(H6N2)[B]	November 5, 2005	X	X
A/blue-winged teal/TX/B94/2006(H6N1)	November 4, 2006	X	X
A/blue-winged teal/TX/B15/2006(H6N1/4)	November 4, 2006	X	X
A/green-winged teal/TX/94/2006(H6N2)	September 23, 2006	X	X
A/blue-winged teal/TX/B102/2006(H6N8)	November 4, 2006	X	
A/blue-winged teal/TX/196/2006(H6N1)	November 4, 2006	X	X
A/blue-winged teal/TX/235/2006(H6N5)	November 4, 2006	X	
A/mottled duck/TX/264/2006(H6N5)	November 4, 2006	X	
A/blue-winged teal/TX/392/2006(H6N6)	November 4, 2006	X	
A/blue-winged teal/TX/499/2006(H6N1)	November 11, 2006	X	X
A/fulvous tree duck/TX/521/2006(H6N1)	November 11, 2006	X	X
A/blue-winged teal/TX/981/2007(H6N1)	September 29, 2007	X	X
A/northern shoveler/TX/1115/2007(H6N2)	November 3, 2007	X	X
A/mottled duck x mallard/TX/1337/2007(H6N8)	November 3, 2007	X	
A/northern shoveler/TX/1901/2007(H6N2)	December 8, 2007	X	X
A/gadwall/TX/2104/2007(H6N1)	December 15, 2007	X	X
A/green-winged teal/TX/1254/2007(H7N3)	January 28, 2007	X	X
A/blue-winged teal/TX/470/2007(H7N1)	September 22, 2007	X	X
A/blue-winged teal/TX/565/2007(H7N7)	September 22, 2007	X	
A/blue-winged teal/TX/854/2007(H7N7)	September 29, 2007	X	
A/blue-winged teal/TX/980/2007(H7N1/4)	September 29, 2007	X	X
A/blue-winged teal/TX/1081/2007(H7N7)	November 3, 2007	X	
A/blue-winged teal/TX/1199/2007(H7N4)	November 3, 2007	X	X
A/green-winged teal/TX/1215/2007(H7N1/4)	November 3, 2007	X	X
A/blue-winged teal/TX/1251/2007(H7N7)	November 3, 2007	X	
A/blue-winged teal/TX/1581/2007(H7N7)	November 10, 2007	X	

Table 16. cont.

Isolate	Date of collection	Gene segment	
		HA	NA
A/green-winged teal/TX/2265/2008(H7N3)	January 12, 2008	X	X
A/cinnamon teal/TX/2301/2008(H7N3)	January 12, 2008	X	X
A/northern shoveler/TX/281/2008(H7N2)	November 8, 2008	X	X

[A]X: indicates that the specified gene from the isolate was included in the study.
[B]Serotyped as an N2, molecular sequence analysis identified an N1.

Table 17. Primers used in this study.

Target	Code	Primer	Sequence (5' to 3")	Nucleotide position	Source
Amplification					
H5	BL 1529	HA_av_H5_M13_1F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGGGT	1	JCV[A]
	BL 1530	HA_av_H5_M13_527F	TGTAAAACGACGGCCAGTTGGYTBATCAARAAG	527	JCV
	BL 1533	HA_av_H5_M13_935F	TGTAAAACGACGGCCAGTATAAAYTCHAGYATGCC	935	JCV
	BL 1558	HA_av_H5_M13_762R	CAGGAAACAGCTATGACCTCCATYCTTCCACTTTGYCC	762	JCV
	BL 1559	HA_av_H5_M13_1139R	CAGGAAACAGCTATGACCCCATKCCYTGCCAYCCYCC	1139	JCV
	BL 1562	HA_av_H5_M13_1802R	CAGGAAACAGCTATGACCAGTAGAAACWAGGGTGTTTT	1802	JCV
	BL-1587	H5 999F+M13	TGT AAA ACG ACG GCC AGT CGG ACA AAC TGG T	999	IN-HOUSE
	BL-1588	H5 979F+M13	TGT AAA ACG ACG GCC AGT CAA ACT GGT CCT TGC	979	IN-HOUSE
H6	BL-1500	H6 HA F		START	IN-HOUSE
	SR-2021	Bm-NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	END	Hoffman, 2001
H7	BL-1535	HA_av_H7_M13_1BF	TGTAAAACGACGGCCAGTAGCAAAAGCAGGGGATACA	1	JCV
	BL-1538	HA_av_H7_M13_630F	TGTAAAACGACGGCCAGTTHTATGGRAGTGGRA	630	JCV
	BL-1539	HA_av_H7_M13_995F	TGTAAAACGACGGCCAGTYTGGCWACAGGRATGA	995	JCV
	BL-1565	HA_av_H7_M13_811R	CAGGAAACAGCTATGACCGGRGCTATRAADGCYCCATTGA	811	JCV

Table 17. cont.

Target	Code	Primer	Sequence (5' to 3")	Nucleotide position	Source
	BL-1568	HA_av_H7_M13_1221R	CAGGAAACAGCTATGACCTGATCWATTGCHGAYTGRGTGC	1221	JCV
	BL-1569	HA_av_H7_M13_1777R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTTT	1777	JCV
NA	BL 1512	NA_av_N1_4_5_7_8_M13_926F	TGTAAAACGACGGCCAGTAGRGAYAAYTGG	926	JCV
	BL 1513	NA_av_N1_4_M13_1BF	TGTAAAACGACGGCCAGTAGCAAAAGCAGGAGWTT	1	JCV
	BL 1514	NA_av_N1_4_M13_517F	TGTAAAACGACGGCCAGTYTRATGAGYTGYCC	517	JCV
	BL 1515	NA_av_N2_7_M13_1F	TGTAAAACGACGGCCAGTAGCAAAAGCAGG	1	JCV
	BL 1516	NA_av_N2_M13_580F	TGTAAAACGACGGCCAGTTDGSRTGGTCYAG	580	JCV
	BL 1517	NA_av_N2_M13_926F	TGTAAAACGACGGCCAGTAGRGAYAAYTGG	926	JCV
	BL 1518	NA_av_N2_M13_973F	TGTAAAACGACGGCCAGTTGTGTGYTCAGGRCTYGTTGG	973	JCV
	BL 1519	NA_av_N3_M13_1CF	TGTAAAACGACGGCCAGTAGCAAAAGCAGGTGCGARATG	1	JCV
	BL 1520	NA_av_N3_M13_409F	TGTAAAACGACGGCCAGTTGGTCYTTTGCHCTHGCNCAAGG	409	JCV
	BL 1521	NA_av_N3_M13_445F	TGTAAAACGACGGCCAGTACHAARCAYAGCAATGG	445	JCV
	BL 1522	NA_av_N3_M13_1060F	TGTAAAACGACGGCCAGTGGAGTBAARGGDTTTGG	1060	JCV
	BL 1523	NA_av_N3_M13_1091BF	TGTAAAACGACGGCCAGTGAYGTRTGGYTVGG	1091	JCV
	BL 1524	NA_av_N7_M13_608F	TGTAAAACGACGGCCAGTGGRAATAATGAYAAYGCHACAGC	608	JCV

Table 17. cont.

Target	Code	Primer	Sequence (5' to 3")	Nucleotide position	Source
	BL 1525	NA_av_N1_4_M13_902F	TGTAAAACGACGGCCAGTGRGAYAAYTGGCRBGG	902	JCV
	BL 1526	NA_av_N3_M13_449BF	TGTAAAACGACGGCCAGTCAYAGCAATGGRAC	449	JCV
	BL 1541	NA_av_N1_4_5_7_8_M13_1460R	CAGGAAACAGCTATGACCAGTAGAAACAAGGAGTTTTTT	1460	JCV
	BL 1542	NA_av_N1_4_M13_679R	CAGGAAACAGCTATGACCTTATGCCATTGTATTTCARTACWGCHACAGC	679	JCV
	BL 1543	NA_av_N1_4_M13_1192R	CAGGAAACAGCTATGACCCCATTTGGATCCCAAAYCATYTCAAANCC	1192	JCV
	BL 1544	NA_av_N2_7_M13_795R	CAGGAAACAGCTATGACCCCTGATGCACTTCCATCMGTCATYACHAC	795	JCV
	BL 1545	NA_av_N2_M13_1128R	CAGGAAACAGCTATGACCCATTGTCAAAGGCCCANCCYTTYACTCC	1128	JCV
	BL 1546	NA_av_N2_M13_1460R	CAGGAAACAGCTATGACCAGTAGAAACAAGGAGTTTTTT	1460	JCV
	BL 1547	NA_av_N3_M13_581R	CAGGAAACAGCTATGACCCCRTCRAARCARCTRCTRCTYGACCA	581	JCV
	BL 1548	NA_av_N3_M13_631R	CAGGAAACAGCTATGACCCACTBGCATCATTRTCRTTYCC	631	JCV
	BL 1549	NA_av_N3_M13_1165R	CAGGAAACAGCTATGACCATCCANCCHTCTGHRACTTTGAT	1165	JCV
	BL 1550	NA_av_N3_M13_1233R	CAGGAAACAGCTATGACCTCATTGTTKGAMACHARTGTYTGTG	1233	JCV
	BL 1551	NA_av_N3_M13_1455R	CAGGAAACAGCTATGACCAGTAGAAACAAGGTGCTTTTT	1455	JCV
	BL 1552	NA_av_N7_M13_1083R	CAGGAAACAGCTATGACCAAMCCGAAYCCYTTBACYC	1083	JCV
	BL 1553	NA_av_N1_4_M13_1460R	CAGGAAACAGCTATGACCAGTAGAAACAAGGAGTTTTT	1460	JCV

Table 17. cont.

Target	Code	Primer	Sequence (5' to 3")	Nucleotide position	Source
	BL 1554	NA_av_N3_M13_1233R	CAGGAAACAGCTATGACCTCATTGTTKGAMACHARTGTYTGTG	1233	JCV
HA	BL 1555	HA_av_M13_1761R	CAGGAAACAGCTATGACCAGTAGAAACAAGGGTRTTTT	1761	JCV
	BL 1556	HA_av_M13_639R	CAGGAAACAGCTATGACCGGAGGATGRTGYACNCCCCA	639	JCV
	BL 1557	HA_av_M13_1780R	CAGGAAACAGCTATGACCAGTAGAAACWAGGGTRTTTT	1780	JCV
	BL 1558	HA_av_H5_M13_762R	CAGGAAACAGCTATGACCTCCATYCTTCCACTTTGYCC	762	JCV
	BL 1559	HA_av_H5_M13_1139R	CAGGAAACAGCTATGACCCCATKCCYTGCCAYCCYCC	1139	JCV
	BL 1527	HA_av_M13_1F	TGTAAAACGACGGCCAGTAGCAAAAGCAGGGG	1	JCV
	BL 1528	HA_av_M13_1135F	TGTAAAACGACGGCCAGTTTYGGNGCNATWGC	1135	JCV
Sequencing					
M13 reverse	BL 1570	M13_R	CAGGAAACAGCTATGACC		
M13 forward	BL 1540	M13_F	TGTAAAACGACGGCCAGT		
H6	BL-1503	H6 1.3	ATC CAA CCC TCT ATG GTG C	250	IN-HOUSE
	BL-1504	H6 2.3	AAG GGG CAA TTA GAT TTC C	800	IN-HOUSE
	BL-1505	H6 3.3	TAA GTT CCT TTA ATT ACT G	540	IN-HOUSE
	BL-1506	H6 4.3	TGG GAT AGT CAT AGG TAC C	1510	IN-HOUSE
	BL-1507	H6 5.3	TTA TTT GTA ATT CCG TC	1180	IN-HOUSE

Table 17. cont.

Target	Code	Primer	Sequence (5' to 3")	Nucleotide position	Source
	BL-1508	H6 1.5	GAC AAT GCT AAT GAT CTA G	1430	IN-HOUSE
	BL-1509	H6 2.5	GCT CAG GAT ATG CAG CAG A	1140	IN-HOUSE
	BL-1510	H6 3.5	GAG ATT TGA GAT GTT TCC C	400	IN-HOUSE
	BL-1511	H6 4.5	GAA ATT GCA GCA AGA CC	700	IN-HOUSE

[A]J. Craig Venter Institute JCVI primer sets: Avian T19 internal segments and NA primers and Avian T20 HA segment primers (http://gsc.jcvi.org/projects/msc/influenza/infl_a_virus/primers.shtml)

5.2.3. Selection of sequences for analysis

The HA gene from eight H5, 16 H6 and 13 H7 and the NA gene of nine N1, nine N2, six N3, and three N4 AIVs isolated from hunter-harvested waterfowl during a multiyear surveillance study along the Texas Mid-Coast (Chanpters II and III) were compared to those available in public databases.

Homologous sequences were selected from GenBank and the Influenza Research Database for analysis and initial alignments were performed within the Avian Influenza Resources available through NCBI (http://ncbi.nlm.nih.gov/genomes/FLU/FLU.html) using MUSCLE-based techniques (Edgar, 2004). Further manual alignments were conducted using MacClade 4 (Release Version 4.08, Sinauer Associates, Sunderland, MA). Protein coding regions were determined and only the open reading frames (ORF) were utilized for analyses. Initially, > 100 sequences from the public databases were compared to our sequences and data sets were subsequently reduced by constructing trees using neighbor-joining methods in MacVector (v. 10.5.1, MacVector, Inc, Cary, NC) and limiting to single taxa representative of close relationships. Additionally, identical taxa and taxa representing a single outbreak were removed or reduced to a single taxa. In cases where the decision to keep a taxa was between poultry or waterfowl, the waterfowl taxa was kept. Final data sets consisted of 43 H5 taxa, 44 H6 taxa, 52 H7 taxa, 41 N1 taxa, 39 N2 taxa, 45 N3 taxa, and 24 N4 taxa each were used for further phylogenetic analysis.

5.2.4. Data analysis

Pairwise distance matrices were calculated for nucleotide sequences and deduced amino acid sequences using Mega version 4 software (Tamura et al., 2007) and percent similarities were calculated. For each gene segment sequenced, the most similar strain was identified by data searching of the open reading frame region at both nucleotide and deduced amino acid sequence using BLASTN or BLASTP, respectively.

Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) methods. MP bootstrapping analysis using a full heuristic algorithm and PAUP program (Swofford, 2002) were used to search for the best trees from 100 replicates. Sequences were added randomly and tree-bisection reconnection was applied for branch swapping during the MP tree search.

For ML analyses, the general time reversible (GTR) model with gamma distribution of rate variation was implemented. ML analyses were conducted using Randomized Axelerated Maximum Likelihood (RAxML) (Stamatakis et al., 2008) and Genetic Algorithm for Rapid Likelihood Inference (GARLI; Zwickl, 2006). For RAxML and GARLI, 100 bootstrap inferences were performed.

RaxML, GARLI, and PAUP were utilized through the CIPRES website (SDSC - UC San Diego, MC 0505 - 9500 Gilman Drive - La Jolla, CA 92093-0505). All tree topologies were visualized with FigTree (v1.2.2) (http://tree.bio.ed.ac.uk/software/figtree/).

5.3. Results

5.3.1. Nucleotide and amino acid similarities of select isolates

Nucleotide and amino acid similarities of the Texas isolates are presented in Tables 18–23. In general, within their respective categories (i.e., H5, H6, H7, N1, N2, N3, and N4) the Texas isolates were 92–100% similar in nucleotides and 94–100% similar in amino acid translations.

Similarity for all H5 sequences examined varied from 86.5–99.5% and 89.0–99.8% at the nucleotide and amino acid levels, respectively (data not shown). The H6 sequences were 77.1–99.0% and 84.4–99.8% similar at the nucleotide and amino acid levels, respectively. The H7 isolates were 88.5–99.0% similar in nucleotide sequence and 92.5–99.8% similar at the protein level. The N1 isolates were 90.0–99.1% and 96.3–100% similar at the nucleotide and amino acid levels, respectively. The N2 isolates were 82.1–99.5% similar in nucleotide sequence and 84.6–99.8% similar in amino acids. The N3 nucleotide sequences were 88.5–99.1% similar and the amino acids were 96.3–99.6% similar. The N4 sequences were 88.4–96.1% similar in nucleotides and 97.3–99.3% similar in amino acids.

Almost all HA and NA sequences and deduced amino acid sequences from the new Texas isolates reported here matched other North American isolates, with a few exceptions. The deduced amino acid sequence from seven of the sixteen H6 isolates more closely matched those of a duck from Hokkaido, Japan (Table 18).

Table 18. Comparison of nucleotide and amino acid similarity of hemagglutinin and neuraminidase genes from wild bird origin AIVs listed in Table 16 to those listed in public databases.

Gene	Isolate	Isolate with closest nucleotide similarity	%	Isolate with closest amino acid similarity	%
H5	A/blue-winged teal/TX/1087/2007(H5N2)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47563 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/green-winged teal/TX/1207/2007(H5N2)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47563 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/northern shoveler/TX/1210/2007(H5N3)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	98	ACX55290 A/mallard/British Columbia/07826/2005(H5N2)	98
	A/blue-winged teal/TX/1402/2007(H5N2)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47563 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/blue-winged teal/TX/1459/2007(H5N3)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47563 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/blue-winged teal/TX/1473/2007(H5N3)	CY034173 A/cinnamon teal/CA/HKWF1111/2007(H5N7)	97	ABB87042 A/mallard duck/ALB/57/1976(H5N2)	97
	A/northern shoveler/TX/1578/2007(H5N2)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47563 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/northern shoveler/TX/2003/2007(H5N2)	CY033444 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47563 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
H6	A/green-winged teal/TX/G39/2005(H6N2)	GU051393 A/northern pintail/TX/184/2002(H6N4)	98	ACZ48444 A/northern pintail/TX/184/2002(H6N4)	99
	A/blue-winged teal/TX/B94/2006(H6N1)	CY032884 A/northern shoveler/CA/HKWF115/2007(H6N1)	99	ACQ83120 A/northern pintail/CA/HKWF440/2007(H6N1)	99
	A/blue-winged teal/TX/B15/2006(H6N1/4)	CY032884 A/northern shoveler/CA/HKWF115/2007(H6N1)	99	ACQ83120 A/northern pintail/CA/HKWF440/2007(H6N1)	99
	A/green-winged teal/TX/94/2006(H6N2)	CY032884 A/northern shoveler/CA/HKWF115/2007(H6N1)	99	ACQ83120 A/northern pintail/CA/HKWF440/2007(H6N1)	99
	A/blue-winged teal/TX/B102/2006(H6N8)	CY021861 A/blue-winged teal/Ohio/1387/2005(H6N2)	99	ABQ01256 A/blue-winged teal/Ohio/1387/2005(H6N2)	99

Table 18. cont.

Gene	Isolate	Isolate with closest nucleotide similarity	%	Isolate with closest amino acid similarity	%
	A/blue-winged teal/TX/196/2006(H6N1)	CY004282 A/shorebird/DE/12/2004(H6N8)	97	ACF47442 A/American wigeon/CA/HKWF1174/2007(H6N1)	97
	A/blue-winged teal/TX/235/2006(H6N5)	CY032704 A/American wigeon/CA/HKWF371/2007(H6N5)	99	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/mottled duck/TX/264/2006(H6N5)	CY033420 A/American wigeon/CA/HKWF42/2007(H6N1)	99	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/blue-winged teal/TX/392/2006(H6N6)	CY032900 A/American wigeon/CA/HKWF295/2007(H6N5)	99	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/blue-winged teal/TX/499/2006(H6N1)	CY032704 A/American wigeon/CA/HKWF371/2007(H6N5)	99	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/fulvous tree duck/TX/521/2006(H6N1)	CY032704 A/American wigeon/CA/HKWF371/2007(H6N5)	98	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/blue-winged teal/TX/981/2007(H6N1)	CY032704 A/American wigeon/CA/HKWF371/2007(H6N5)	99	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/northern shoveler/TX/1115/2007(H6N2)	CY032900 A/American wigeon/CA/HKWF295/2007(H6N5)	99	BAF47399 A/duck/Hokkaido/228/2003(H6N8)	99
	A/mottled duck x mallard/TX/1337/2007(H6N8)	CY039612 A/northern pintail/CA/HKWF440/2007(H6N1)	98	ACQ83120 A/northern pintail/CA/HKWF440/2007(H6N1)	98
	A/northern shoveler/TX/1901/2007(H6N2)	CY039612 A/northern pintail/CA/HKWF440/2007(H6N1)	98	ACQ83120 A/northern pintail/CA/HKWF440/2007(H6N1)	98
	A/gadwall/TX/2104/2007(H6N1)	CY033356 A/American wigeon/CA/HKWF1174/2007 (H6N1)	99	ACF47442 A/American wigeon/CA/HKWF1174/2007(H6N1)	99
H7	A/green-winged teal/TX/1254/2007(H7N3)	CY033380 A/mallard/CA/HKWF1971/2007(H7N7)	99	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/blue-winged teal/TX/470/2007(H7N1)	CY039580 A/northern shoveler/CA/HKWF1026/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99

Table 18. cont.

Gene	Isolate	Isolate with closest nucleotide similarity	%	Isolate with closest amino acid similarity	%
H7	A/blue-winged teal/TX/565/2007(H7N7)	GU186474 A/northern shoveler/NC/6412-052/2005(H7N6)	98	ACV41603 A/American green-winged teal/CA/44287-713/2007(H7N3)	99
	A/blue-winged teal/TX/854/2007(H7N7)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/blue-winged teal/TX/980/2007(H7N1/4)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/blue-winged teal/TX/1081/2007(H7N7)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/blue-winged teal/TX/1199/2007(H7N4)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/green-winged teal/TX/1215/2007(H7N1/4)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/blue-winged teal/TX/1251/2007(H7N7)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/blue-winged teal/TX/1581/2007(H7N7)	CY047011 A/American green-winged teal/CA/28855/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/green-winged teal/TX/2265/2008(H7N3)	CY039580 A/northern shoveler/CA/HKWF1026/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/cinnamon teal/TX/2301/2008(H7N3)	CY039580 A/northern shoveler/CA/HKWF1026/2007(H7N3)	98	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
	A/northern shoveler/TX/281/2008(H7N2)	GU186474 A/northern shoveler/NC/6412-052/2005(H7N6	97	ACK43671 A/northern pintail/CA/44387-486/2008(H7N3)	99
N1	A/blue-winged teal/TX/B15/2006(H6N1/4)	EF607895 A/black duck/NC/674-694/2006(H5N1)	99	ABU95355 A/black duck/NC/674-694/2006(H5N1)	99
	A/blue-winged teal/TX/B94/2006(H6N1)	CY032886 A/northern shoveler/CA/HKWF115/2007(H6N1)	99	ACE76616 A/northern shoveler/CA/HKWF383/2007(H6N1)	100

Table 18. cont

Gene	Isolate	Isolate with closest nucleotide similarity	%	Isolate with closest amino acid similarity	%
N1	A/blue-winged teal/TX/196/2006(H6N1)	EF607895 A/black duck/NC/674-694/2006(H5N1)	99	ACQ83133 A/northern shoveler/CA/HKWF569/2007(H3N1)	99
	A/blue-winged teal/TX/499/2006(H6N1)	CY033422 A/American wigeon/CA/HKWF42/2007(H6N1)	99	ABQ43792 A/mallard/ON/499/2005(H5N1)	99
	A/fulvous tree duck/TX/521/2006(H6N1)	CY033422 A/American wigeon/CA/HKWF42/2007(H6N1)	99	ABQ43792 A/mallard/ON/499/2005(H5N1)	99
	A/blue-winged teal/TX/981/2007(H6N1)	CY045393 A/pintail/ALB/21/2006(H1N1)	99	ACV A/pintail/ALB/21/2006(H1N1)	99
	A/gadwall/TX/2104/2007(H6N1)	CY032886 A/northern shoveler/CA/HKWF115/2007(H6N1)	99	ACE76616 A/northern shoveler/CA/HKWF383/2007(H6N1)	99
	A/blue-winged teal/TX/470/2007(H7N1)	CY042073 A/mallard duck/MN/Sg-00105/2007(H6N1	99	ACQ82827 A/northern pintail/Interior Alaska/1/2007(H1N1)	99
	A/green-winged teal/TX/G39/2005(H6N2)[A]	CY005094 A/mallard/ALB/34/2001(H7N1)	99	ABG88259 A/mallard/OH/56/1999(H1N1)	99
N2	A/blue-winged teal/TX/1087/2007(H5N2)	CY033446 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/green-winged teal/TX/1207/2007(H5N2)	CY033446 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/blue-winged teal/TX/1402/2007(H5N2)	CY033446 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/northern shoveler/TX/1578/2007(H5N2)	CY033446 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/northern shoveler/TX/2003/2007(H5N2)	CY033446 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/green-winged teal/TX/94/2006(H6N2)	CY045321 A/northern pintail/Saskatchewan/22910/2007(H3N2)	99	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99

Table 18. cont.

Gene	Isolate	Isolate with closest nucleotide similarity	%	Isolate with closest amino acid similarity	%
N3	A/northern shoveler/TX/1115/2007(H6N2)	CY045321 A/northern pintail/Saskatchewan/22910/2007(H3N2)	98	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/northern shoveler/TX/1901/2007(H6N2)	CY045321 A/northern pintail/Saskatchewan/22910/2007(H3N2)	98	ACF47565 A/American green-winged teal/CA/HKWF609/2007(H5N2)	99
	A/northern shoveler/TX/281/2008(H7N2)	CY054538 A/blue-winged teal/MN/Sg-00798/2008(H4N2)	99	ABL75577 A/green-winged teal/OH/203/1998(H6N2)	99
	A/northern shoveler/TX/1210/2007(H5N3)	GU052839 A/mallard/Montana/458328-5/2006(H5N3)	99	ACZ45101 A/mallard/Montana/45623 /2006 (H5N3)	99
	A/blue-winged teal/TX/1459/2007(H5N3)	CY039598 A/northern shoveler/CA/HKWF2031/2008(H7N3)	99	ACQ83023 A/green-winged teal/CA/AKS1370/2008(H7N3)	99
	A/blue-winged teal/TX/1473/2007(H5N3)	CY053791 A/northern shoveler/CA/JN587/2006(H10N3)	99	ADA82082 A/northern shoveler/CA/JN587/2006(H10N3)	99
	A/green-winged teal/TX/1254/2007(H7N3)	CY039598 A/northern shoveler/CA/HKWF2031/2008(H7N3)	99	ACQ83023 A/green-winged teal/CA/AKS1370/2008(H7N3)	99
	A/green-winged teal/TX/2265/2008(H7N3)	CY039582 A/northern shoveler/CA/HKWF1026/2007(H7N3)	99	ADA82082 A/northern shoveler/CA/JN587/2006(H10N3)	99
	A/cinnamon teal/TX/2301/2008(H7N3)	CY039582 A/northern shoveler/CA/HKWF1026/2007(H7N3)	99	ADA82082 A/northern shoveler/CA/JN587/2006(H10N3)	99
N4	A/blue-winged teal/TX/980/2007(H7N1/4)	CY039590 A/northern shoveler/CA/HKWF1204/2007(H8N4)	96	ACF74214 A/northern shoveler/CA/HKWF1203/2007(H8N4)	99
	A/green-winged teal/TX/1215/2007(H7N1/4)	CY039590 A/northern shoveler/CA/HKWF1204/2007(H8N4)	95	ACF74214 A/northern shoveler/CA/HKWF1203/2007(H8N4)	99
	A/blue-winged teal/TX/1199/2007(H7N4)	CY039590 A/northern shoveler/CA/HKWF1204/2007(H8N4)	95	ACF74214 A/northern shoveler/CA/HKWF1203/2007(H8N4)	99

[A]Serotyped as an N2, molecular sequence analysis identified an N1.

Table 19. Percent pairwise similarity of nucleotide and amino acid sequences among H5 AIV isolates listed in table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3	4	5	6	7	8
1	A/blue-winged teal/ TX/1087/2007(H5N2)		**100.0**	**98.9**	**99.6**	**99.6**	**99.6**	**97.3**	**99.5**
2	A/green-winged teal/ TX/1207/2007(H5N2)	99.6		**98.9**	**99.6**	**99.6**	**99.6**	**97.3**	**99.5**
3	A/northern shoveler/ TX/1210/2007(H5N3)	98.9	99.0		**98.6**	**98.6**	**98.6**	**97.0**	**98.4**
4	A/northern shoveler/ TX/1578/2007(H5N2)	99.3	99.4	98.6		**99.3**	**99.3**	**97.0**	**99.1**
5	A/blue-winged teal/ TX/1402/2007(H5N2)	99.3	99.4	98.6	99.1		**99.3**	**97.0**	**99.1**
6	A/blue-winged teal/ TX/1459/2007(H5N3)	99.5	99.6	98.8	99.4	99.2		**97.0**	**99.1**
7	A/blue-winged teal/ TX/1473/2007(H5N3)	94.9	95.0	95.0	94.7	94.8	95.0		**96.8**
8	A/northern shoveler/ TX/2003/2007(H5N2)	99.3	99.4	98.6	99.1	99.5	99.2	94.7	

Table 20. Percent pairwise similarity of nucleotide and amino acid sequences among H6 AIV isolates listed in Table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	A/gadwall/TX/2104/2007(H6N1)		**97.2**	**98.6**	**98.6**	**98.4**	**97.2**	**98.8**	**98.6**	**98.9**	**98.6**	**97.9**	**97.5**	**97.2**	**98.2**	**98.8**	**98.6**
2	A/mottled duck x mallard/TX/1337/2007(H6N8)	97.2		**97.9**	**98.6**	**98.4**	**99.8**	**97.7**	**98.2**	**97.9**	**98.6**	**96.8**	**96.8**	**96.3**	**97.5**	**98.1**	**97.9**
3	A/blue-winged teal/TX/981/2007(H6N1)	98.0	97.4		**99.3**	**99.1**	**97.9**	**99.5**	**99.6**	**99.6**	**99.3**	**98.6**	**98.6**	**97.9**	**99.3**	**99.5**	**100**
4	A/blue-winged teal/TX/B94/2006(H6N1)	98.3	98.7	98.4		**99.8**	**98.4**	**99.1**	**99.3**	**99.3**	**100**	**98.2**	**98.2**	**97.5**	**98.9**	**99.5**	**99.3**
5	A/green-winged teal/TX/94/2006(H6N2)	98.2	98.8	98.4	99.7		**98.2**	**98.9**	**99.1**	**99.1**	**99.8**	**98.1**	**98.1**	**97.3**	**98.8**	**99.3**	**99.1**
6	A/northern shoveler/ TX/1901/2007(H6N2)	97.2	99.9	97.2	98.6	98.7		**97.7**	**98.2**	**97.9**	**98.4**	**96.8**	**96.8**	**96.3**	**97.5**	**98.1**	**97.9**
7	A/blue-winged teal/TX/235/2006(H6N5)	97.9	97.5	98.9	98.5	98.5	97.5		**99.5**	**99.8**	**99.1**	**98.2**	**98.4**	**97.7**	**99.1**	**99.3**	**99.5**
8	A/mottled duck/ TX/264/2006(H6N5)	97.6	97.4	98.7	98.3	98.3	97.3	99.5		**99.6**	**99.3**	**98.4**	**98.6**	**97.9**	**99.3**	**99.5**	**99.6**
9	A/blue-winged teal/TX/499/2006(H6N1)	97.8	97.3	98.8	98.4	98.4	97.2	99.5	99.8		**99.3**	**98.4**	**98.6**	**97.9**	**99.3**	**99.5**	**99.6**
10	A/blue-winged teal/ TX/B15/2006(H6N1/4)	98.1	98.5	98.2	99.6	99.5	98.5	98.3	98.1	98.2		**98.2**	**98.2**	**97.5**	**98.9**	**99.5**	**99.3**
11	A/blue-winged teal/TX/B102/2006(H6N8)	93.2	93.0	94.4	93.9	93.8	92.9	93.9	93.8	93.8	93.7		**98.4**	**97.3**	**98.1**	**98.4**	**98.6**
12	A/green-winged teal/TX/G39/2005(H6N2)	92.5	92.3	93.3	93.0	92.9	92.2	93.1	92.9	93.0	92.9	92.9		**97.2**	**98.2**	**98.4**	**98.6**
13	A/blue-winged teal/TX/196/2006(H6N1)	96.9	96.3	97.6	97.4	97.3	96.2	97.6	97.4	97.5	97.2	93.2	92.7		**97.5**	**97.9**	**97.9**
14	A/northern shoveler/ TX/1115/2007(H6N2)	97.1	96.8	98.2	97.8	97.8	96.8	98.8	98.6	98.7	97.7	93.6	92.7	96.9		**99.1**	**99.3**
15	A/fulvous tree duck/TX/521/2006(H6N1)	97.8	97.5	98.7	98.4	98.4	97.4	99.6	99.3	99.2	98.2	94.1	93.1	97.5	98.8		**99.5**
16	A/blue-winged teal/TX/392/2006(H6N6)	97.8	97.4	98.8	98.3	98.3	97.3	99.5	99.3	99.2	98.2	93.8	93.1	97.4	99.0	99.3	

Table 21. Percent pairwise similarity of nucleotide and amino acid sequences among H7 AIV isolates listed in Table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	A/blue-winged teal/TX/854/2007(H7N7)		**99.3**	**99.1**	**98.9**	**99.3**	**100**	**99.5**	**99.3**	**99.8**	**99.5**	**100**	**99.3**	**99.1**
2	A/northern shoveler/TX/281/2008(H7N2)	97.9		**98.9**	**98.7**	**99.1**	**99.3**	**99.3**	**99.1**	**99.1**	**99.3**	**99.3**	**99.1**	**98.9**
3	A/blue-winged teal/TX/470/2007(H7N1)	98.4	97.7		**98.7**	**99.1**	**99.1**	**99.3**	**99.1**	**98.9**	**99.3**	**99.1**	**99.5**	**99.3**
4	A/blue-winged teal/TX/565/2007(H7N7)	98.3	98.2	97.9		**98.9**	**98.9**	**99.1**	**98.9**	**98.7**	**99.1**	**98.9**	**98.9**	**98.7**
5	A/blue-winged teal/TX/980/2007(H7N1/4)	98.9	97.9	98.2	98.1		**99.3**	**99.8**	**99.6**	**99.1**	**99.5**	**99.3**	**99.3**	**99.1**
6	A/blue-winged teal/TX/1081/2007(H7N7)	100	97.9	98.4	98.3	98.9		**99.5**	**99.3**	**99.8**	**99.5**	**100**	**99.3**	**99.1**
7	A/blue-winged teal/TX/1199/2007(H7N4)	98.9	97.9	98.0	98.1	99.8	98.9		**99.8**	**99.3**	**99.6**	**99.5**	**99.5**	**99.3**
8	A/green-winged teal/TX/1215/2007(H7N1/4)	98.9	97.9	98.0	98.1	99.8	98.9	99.9		**99.1**	**99.5**	**99.3**	**99.3**	**99.1**
9	A/blue-winged teal/TX/1251/2007(H7N7)	99.8	97.7	98.2	98.2	98.8	99.8	98.8	98.8		**99.3**	**99.8**	**99.1**	**98.9**
10	A/green-winged teal/TX/1254/2007(H7N3)	99.5	98.0	98.5	98.5	99.0	99.5	99.0	99.0	99.3		**99.5**	**99.5**	**99.3**
11	A/blue-winged teal/TX/1581/2007(H7N7)	100	97.9	98.4	98.3	98.9	100	98.9	98.9	99.8	99.5		**99.3**	**99.1**
12	A/green-winged teal/TX/2265/2008(H7N3)	98.3	97.6	99.1	97.7	98.1	98.3	98.0	98.0	98.2	98.5	98.3		**99.8**
13	A/cinnamon teal/TX/2301/2008(H7N3)	98.3	97.6	99.2	97.8	98.0	98.3	97.9	97.9	98.1	98.4	98.3	99.5	

Table 22. Percent pairwise similarity of nucleotide and amino acid sequences among N1 AIV isolates listed in Table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3	4	5	6	7	8	9
1	A/blue-winged teal/TX/470/2007(H7N1)		**97.4**	**97.2**	**97.7**	**98.9**	**97.4**	**97.7**	**97.7**	**97.4**
2	A/blue-winged teal/TX/B94/2006(H6N1)	93.6		**99.4**	**98.5**	**97.7**	**97.9**	**99.1**	**99.1**	**98.7**
3	A/gadwall/TX/2104/2007(H6N1)	93.4	99.4		**98.3**	**97.4**	**97.7**	**98.5**	**98.5**	**98.1**
4	A/blue-winged teal/TX/B15/2006(H6N1/4)	93.3	97.2	96.7		**97.9**	**98.1**	**98.5**	**98.5**	**99.4**
5	A/green-winged teal/TX/G39/2005(H6N2)	97.2	94.3	94.1	94.0		**97.7**	**97.9**	**97.9**	**97.7**
6	A/blue-winged teal/TX/981/2007(H6N1)	93.8	96.7	96.2	95.7	94.5		**97.9**	**97.9**	**97.9**
7	A/blue-winged teal/TX/499/2006(H6N1)	93.1	98.3	97.8	96.8	93.8	96.0		**100**	**98.7**
8	A/fulvous tree duck/TX/521/2006(H6N1)	93.5	98.5	98.0	96.9	94.1	96.2	99.5		**98.7**
9	A/blue-winged teal/TX/196/2006(H6N1)	93.1	97.3	96.7	99.1	94.0	95.6	96.9	97.0	

Table 23. Percent pairwise similarity of nucleotide and amino acid sequences among N2 AIV isolates listed in Table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3	4	5	6	7	8	9
1	A/northern shoveler/TX/281/2008(H7N2)		**94.5**	**94.3**	**94.7**	**94.7**	**95.0**	**95.0**	**95.0**	**95.0**
2	A/northern shoveler/TX/1578/2007(H5N2)	90.6		**98.7**	**99.3**	**98.9**	**98.9**	**99.1**	**99.1**	**98.7**
3	A/blue-winged teal/TX/1087/2007(H5N2)	90.6	99.2		**99.3**	**98.5**	**98.5**	**98.7**	**98.7**	**98.2**
4	A/green-winged teal/TX/1207/2007(H5N2)	90.9	99.5	99.7		**99.1**	**99.1**	**99.3**	**99.3**	**98.9**
5	A/green-winged teal/TX/94/2006(H6N2)	90.7	98.9	98.8	99.1		**99.1**	**98.9**	**98.9**	**98.9**
6	A/northern shoveler/TX/1901/2007(H6N2)	90.7	98.6	98.5	98.8	99.0		**98.9**	**98.9**	**99.8**
7	A/northern shoveler/TX/2003/2007(H5N2)	90.7	99.3	99.6	99.3	98.7	98.5		**100**	**98.7**
8	A/blue-winged teal/TX/1402/2007(H5N2)	90.8	99.3	99.1	99.4	98.8	98.5	99.6		**98.7**
9	A/northern shoveler/TX/1115/2007(H6N2)	90.4	98.1	98.0	98.3	98.5	99.4	98.0	98.0	

Table 24. Percent pairwise similarity of nucleotide and amino acid sequences among N3 AIV isolates listed in Table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3	4	5	6
1	A/northern shoveler/TX/1210/2007(H5N3)		**97.9**	**98.3**	**97.7**	**98.1**	**98.1**
2	A/blue-winged teal/TX/1459/2007(H5N3)	96.0		**99.1**	**98.9**	**98.9**	**99.1**
3	A/blue-winged teal/TX/1473/2007(H5N3)	96.2	97.5		**98.9**	**99.8**	**99.8**
4	A/green-winged teal/TX/1254/2007(H7N3)	96.4	98.9	97.8		**98.7**	**98.9**
5	A/green-winged teal/TX/2265/2008(H7N3)	95.8	97.0	99.1	97.3		**99.6**
6	A/cinnamon teal/TX/2301/2008(H7N3)	95.8	97.0	99.1	97.3	99.4	

Table 25. Percent pairwise similarity of nucleotide and amino acid sequences among N4 AIV isolates listed in Table 16. Nucleotide percent similarity is listed in the lower left bottom and amino acid percent similarity is in the upper right and in bold.

		1	2	3
1	A/blue-winged teal/TX/980/2007(H7N1/4)		**100**	**100**
2	A/green-winged teal/TX/1215/2007(H7N1/4)	99.9		**100**
3	A/blue-winged teal/TX/1199/2007(H7N4)	99.9	100	

5.3.2. Phylogenetic analyses

Representative phylogenetic trees for the H5, H6, H7, N1, N2, N3, and N4 open reading frames are presented in Figs. 4–10. Based on the HA and NA sequences reported here, all new Texas isolates grouped relatively close together indicating similarity for those genes. All except the H6 grouped with other North American isolates. Also, one H5 Texas isolate, A/blue-winged teal/TX/1473/2007(H5N3), grouped away from the seven other H5 isolates, but still grouped with other isolates from North America. One isolate, A/green-winged teal/TX/G39/2005(H6N2), that was identified as an N2 using the neuraminidase inhibition assay was identified as N1 by sequencing, All of the H7 isolates grouped together although the time of collection ranged from January 2007 to November 2008. Similarly, most of the NA subtypes sequenced, N1, N2, N3, and N4, grouped together with a few exceptions. A/northern shoveler/TX/281/2008(H7N2) branched separately from the other N2 isolates, however it was 90% identical in nucleotide sequence and 94-95% similar in amino acid sequence to the others. A/northern shoveler/TX/1210/2007(H5N3) separated phylogenetically from the other Texas isolates and grouped closer to a couple of H5N3 isolates obtained from mallards in Montana. Most all of the isolates were most closely related to isolates from the Pacific flyway—California in particular. There were no consistent patterns in grouping observed in regards to HA and NA combinations, species, or geographic region.

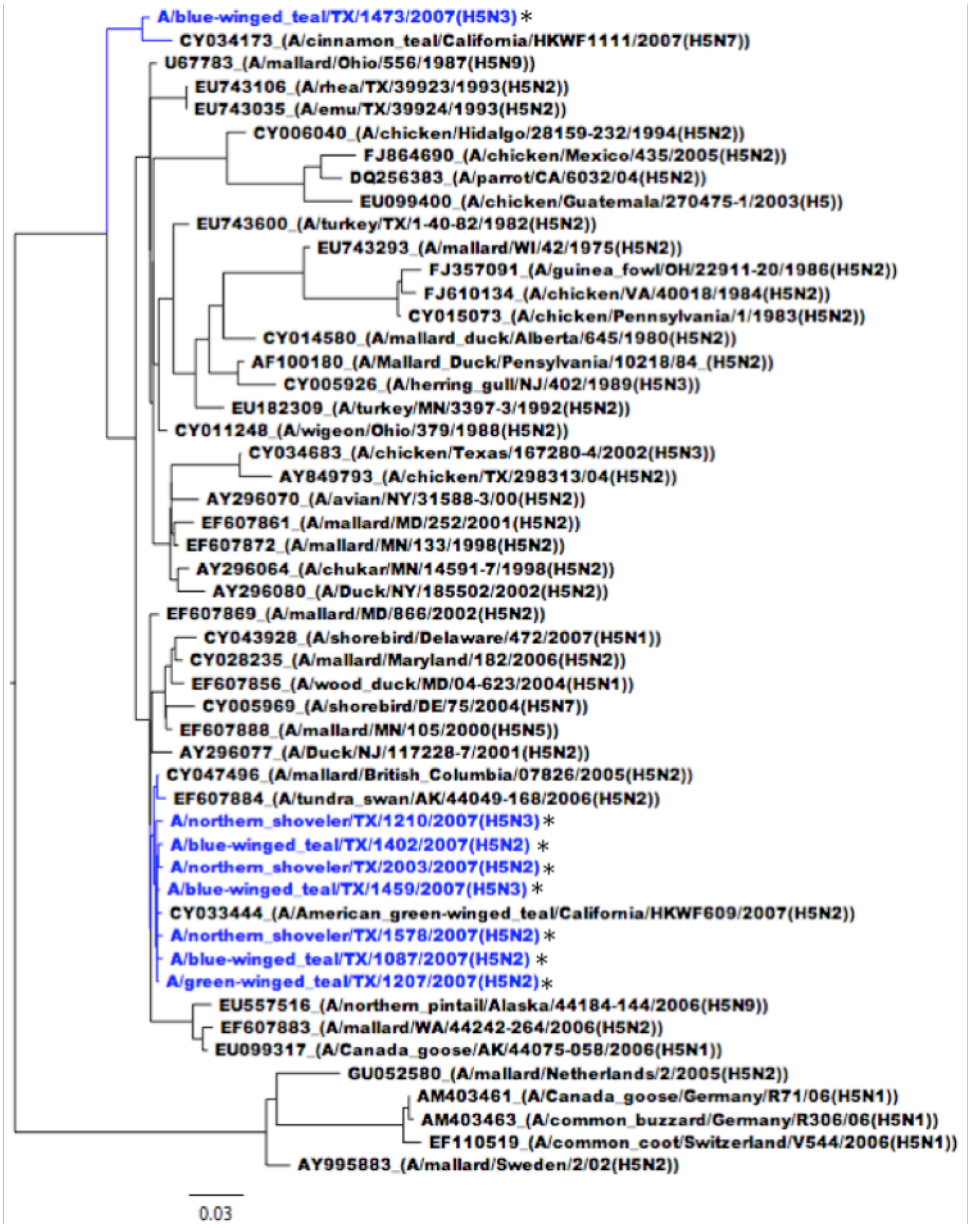

Fig 4. Phylogenetic tree of the nucleotide sequence from the H5 open reading frame of viruses listed in Table 16 and other H5 viruses available in public databases. The tree consists of select sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI using a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

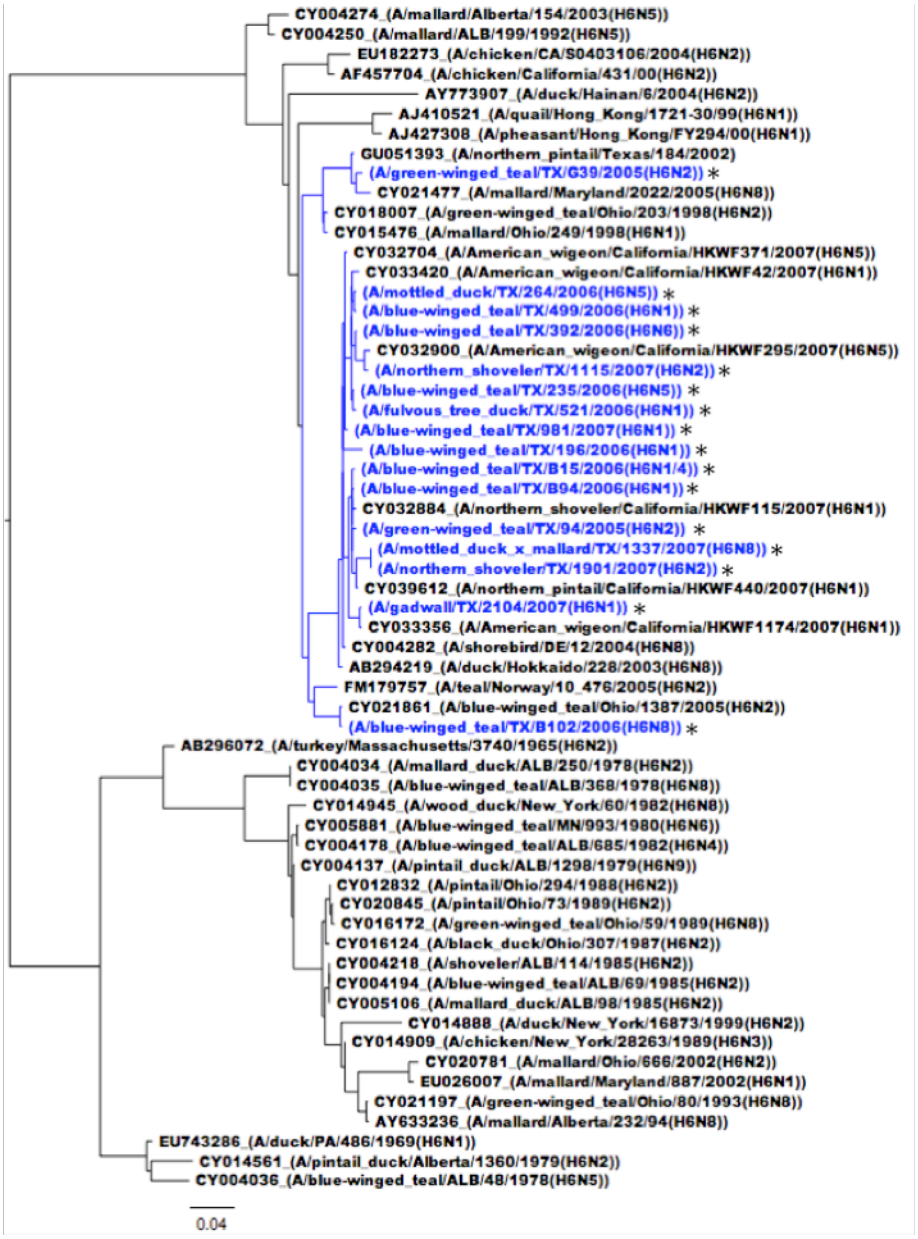

Fig. 5. Phylogenetic tree of the nucleotide sequence from the H6 open reading frame of viruses listed in Table 16 and other H6 viruses available in public databases. The tree consists of sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI using a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

Fig. 6. Phylogenetic tree of the nucleotide sequence from the H7 open reading frame of viruses listed in Table 16 and other H7 viruses available in public databases. The tree consists of sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI using a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

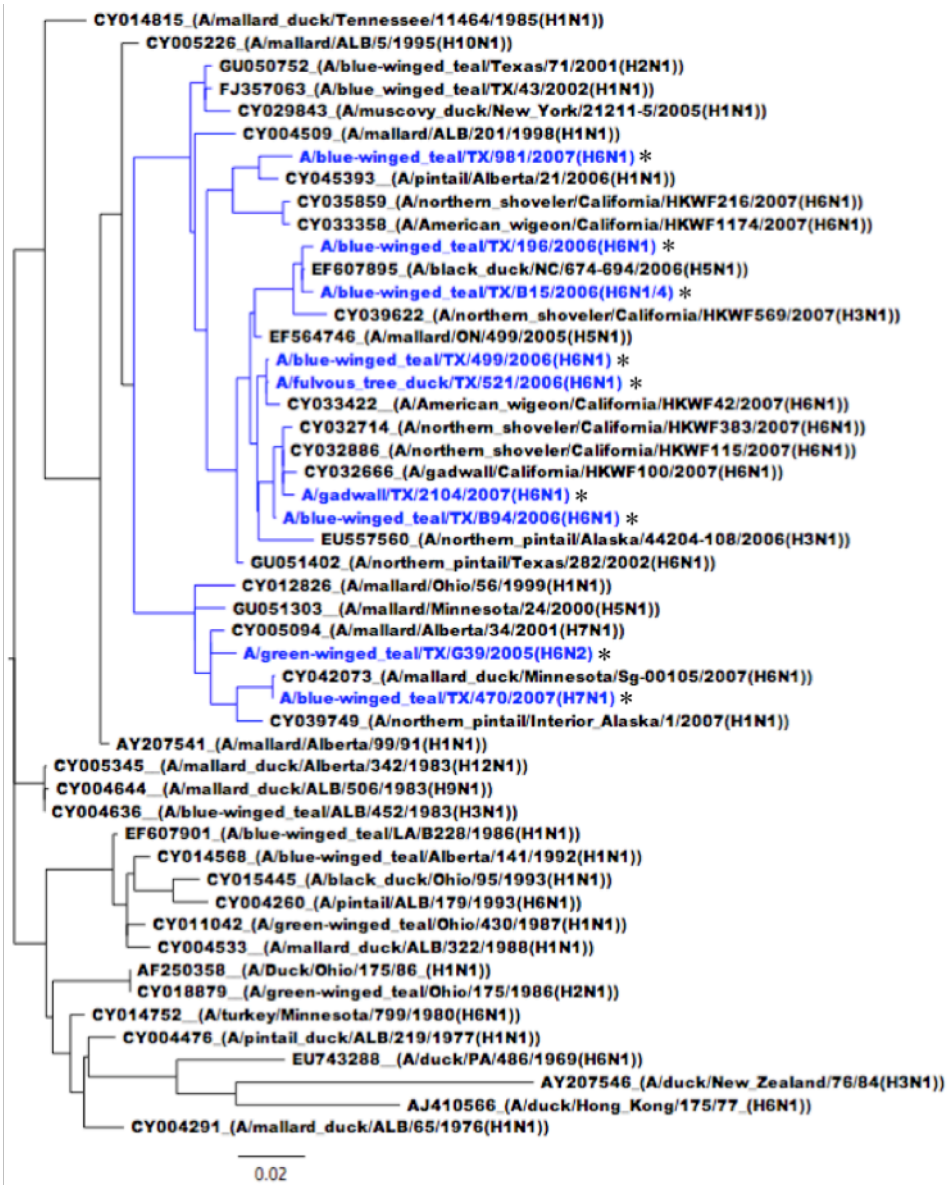

Fig. 7. Phylogenetic tree of the nucleotide sequence from the N1 open reading frame of viruses listed in Table 16 and other N1 viruses available in public databases. The tree consists of sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI using a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

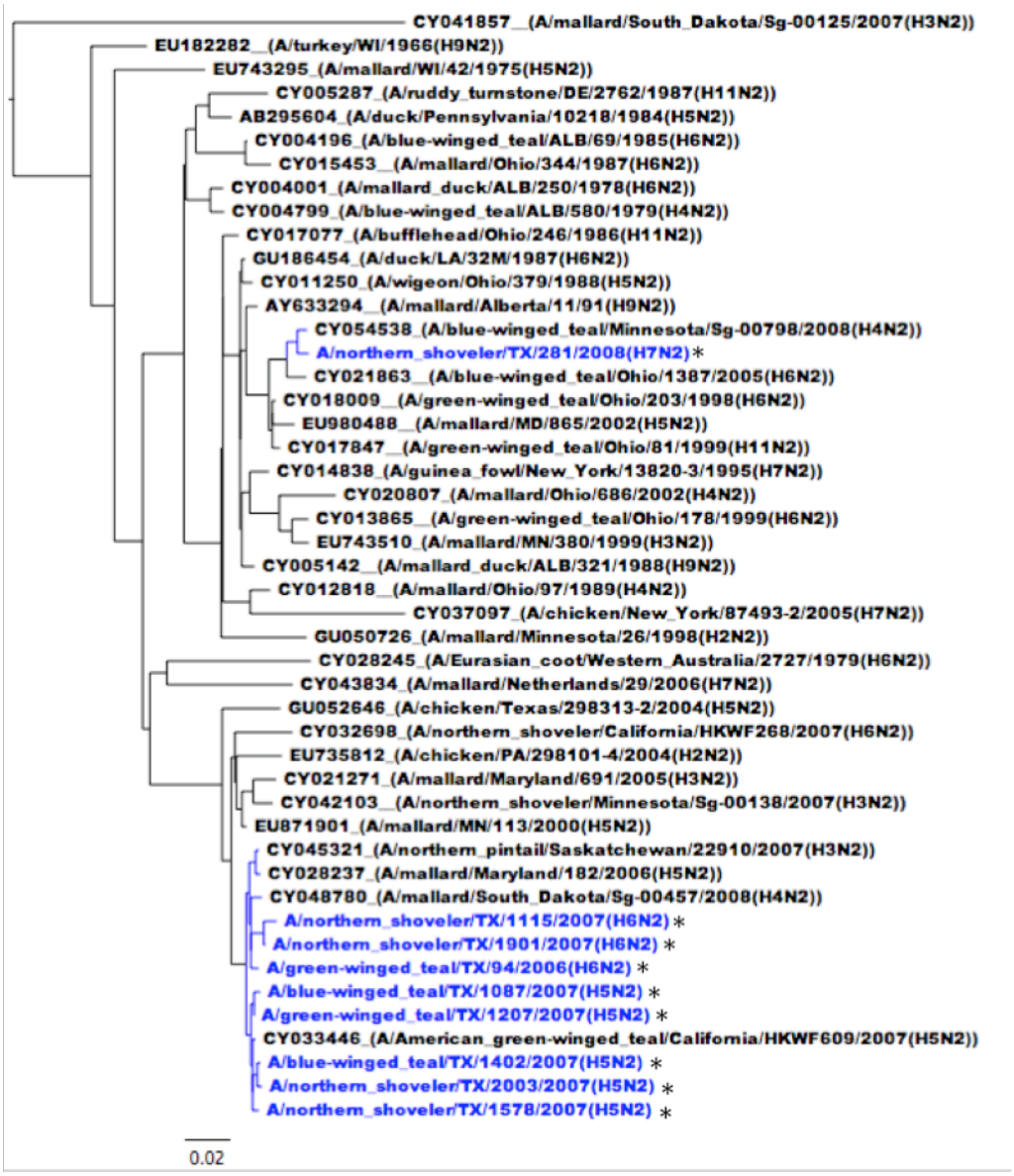

Fig. 8. Phylogenetic tree of the nucleotide sequence from the N2 open reading frame of viruses listed in Table 16 and other N2 viruses available in public databases. The tree consists of sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI with 100 bootstrap replications and a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

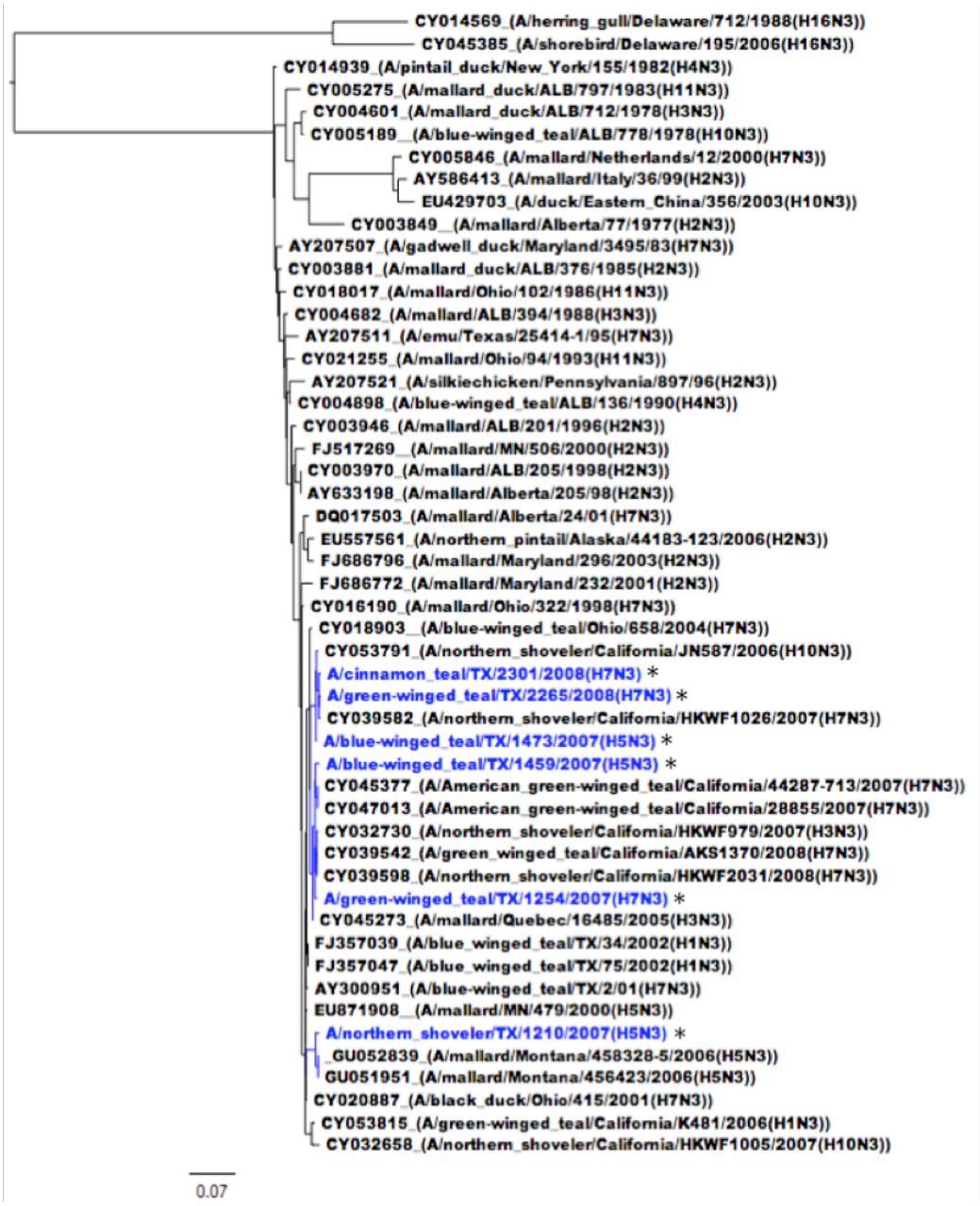

Fig. 9. Phylogenetic tree of the nucleotide sequence from the N3 open reading frame of viruses listed in Table 16 and other N3 viruses available in public databases. The tree consists of sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI using a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

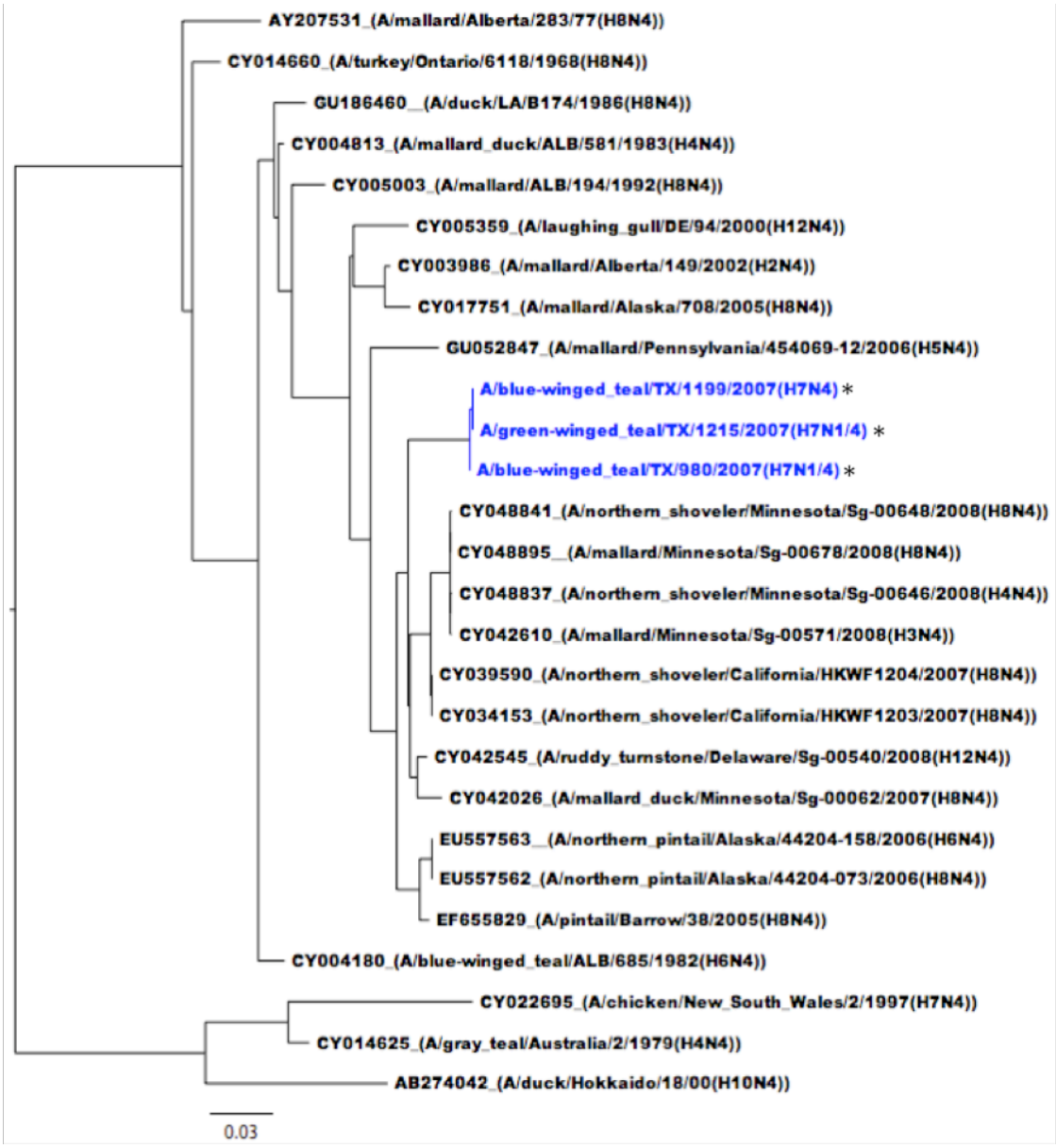

Fig. 10. Phylogenetic tree of the nucleotide sequence from the N4 open reading frame of viruses listed in Table 16 and other N4 viruses available in public databases. The tree consists of sequences from GenBank including North American and Eurasian AIV isolates. The tree was generated in GARLI using a GTR+gamma model of evolution. The new Texas isolates are highlighted in blue and marked with an asterisk (*).

5.4. Discussion

The viruses sequenced as part of this study were low pathogenic AIVs isolated from presumably healthy migratory waterfowl, primarily dabbling ducks. Molecular sequence analysis supported the identification of LPAIV in that no insertions of multiple basic amino acids at the cleavage site were identified. All molecular sequencing returned results similar to the classical subtyping methods of hemagglutination inhibition (HI) and neuraminidase inhibition (NI) assays with the exception of one NA gene. One isolate that was identified as an N2 using classical NI assays was identified as an N1 by molecular sequencing. There are two possible reasons for this discrepancy in classification. First, steric inhibition could be a factor in the NI assay if the reference reagent consisted of the same HA as the isolate. The second could be due to a mixed virus infection and that the N1 was preferentially amplified over the N2; however, this is unlikely because N2 specific primers failed to amplify the genome and no background interference in the sequencing reactions were noted (data not shown).

All of the newly sequenced H5 Texas isolates grouped relatively closely together with one exception, A/blue-winged teal/TX/1473/2007(H5N3). This is not surprising in that most of the isolates were collected within about a month time span and within the same wildlife management area (Ferro unpublished data). Interestingly, the one isolate that was the most different molecularly was collected the same day as five of the eight isolates and was 94–95% identical in nucleotide sequence and 97% similar at the amino acid level to the other Texas isolates. This could be due to co-circulation of influenza viruses of different origins or different evolutionary selection pressures within the host.

AIVs have evolved into two lineages: the Eurasian avian and the North American (Fusaro et al., 2010; Wahlgren et al., 2008). The mechanism by which this separation occurred is not clear and some subtypes have crossed continents. This is particularly true for the H6 subtype. A phylogenetic study published in 2005 described North American origin wild aquatic bird isolates H6 and NS subtype A genes separating into major clades with Eurasian isolates (Spackman et al., 2005). Wahlgren et al. (2008) observed a similar clustering of H6 sequences of North American origin AIV. Their analysis reported a spillover of the Asian H6 and indicated establishment in North America of the Asian H6 gene (Wahlgren et al., 2008). A more recent and extensive study of all H6 sequences available at the time indicated replacement of the North American H6 lineage by the Eurasian H6 lineage (zu Dohna et al., 2009). Sequencing of the H6 isolates herein indicates the same is true in the Central Flyway, in that they are most closely related to H6 isolates from the Pacific Flyway and group with Eurasian H6 lineages. Given the close proximity and the slight overlapping of the Central and Pacific Flyways, the similarity in H6 sequences is not surprising.

Other studies have shown H6 to have a wider host range than other subtypes which could enable this subtype to spread between and within hemispheres (Munster et al., 2007). What is probably more surprising is why this phenomenon has not been observed with other subtypes and why complete viruses from each hemisphere have not crossed over. Perhaps as more viruses are discovered and sequences determined more intercontinental exchanges of AIV genes will be identified. Additionally, gaps in

knowledge still exist in fully understanding migratory patterns of aquatic water birds and how year-to-year variations in environmental conditions affect these patterns.

Many studies have been undertaken to explain evolution in AIVs, most with the goal of predicting characteristics indicative of inter-species transmission potential. Unlike patterns observed with human and chicken influenza type A viruses that show a ladder-like evolutionary pattern (Gorman et al., 1992; Spackman et al., 2005), wild bird origin type A influenza viruses so far have not followed this pattern, thus making predictions difficult (Spackman et al., 2005). We observed similar characteristics with our isolates in that there was no pattern based on time of collection, subtype identified, or species of waterfowl that the samples came from. Given the high rate of mutation that occurs with RNA viruses it is unlikely that these viruses have reached evolutionary stasis (Gorman et al., 1992; Webster et al., 1992). In fact, more recently reports indicate influenza viruses are still undergoing rapid evolutionary changes (Chen and Holmes, 2006; Worobey, 2008). A problem with genetic analyses thus far, particularly with influenza viruses, is that these analyses rely on consensus sequences, which means the nucleotide shown is the most common among all the genomes within a host (Holmes and Grenfell, 2009). Additionally, when analyzing viral isolates, selection has already occurred for the virus that grows best in the isolation system (embryonated chicken eggs in the case of AIV), thereby eliminating sequence information that may be critical to understanding the evolution of influenza viruses under study. Perhaps as newer technologies and techniques are developed these issues will be resolved.

In sum, we sequenced the ORF of three subtypes of hemagglutinin: H5, H6, and H7 and four neuraminidase subtypes: N1, N2, N3, and N4 from isolates obtained from waterfowl on the wintering grounds of the Central Flyway (Chapters X and X). Data herein will contribute to understanding AIVs in their natural reservoir hosts, wild aquatic waterfowl. Further analyses including more HA and NA subtypes and additional genes are planned and will be valuable in understanding AIV ecology and evolution in low prevalence areas, such as the wintering grounds of the Central Flyway.

CHAPTER VI

SUMMARY

Influenza viruses affect a wide range of species from birds to humans and influence the global economy through laborer absenteeism in the case of human infections or culling events in the case of poultry and swine infections. Wild water birds, primarily species of the orders *Charadriiformes* (particularly gulls and shorebirds) and *Anseriformes* (ducks, geese, and swans), are considered the natural reservoir hosts for all type A influenza viruses (Webster et al., 1992). Surveillance and characterization of influenza viruses in reservoir species is essential to our understanding of these viruses, in particular how they persist in nature and change over time. Although studies in areas characterized by low avian influenza virus (AIV) prevalence are inconvenient due to the large sample sizes required to isolate significant numbers of AIVs, such surveys are critical to our understanding of the ecology of influenza viruses and their impact on other species.

The Texas Gulf Coast is a primary wintering ground for migratory waterfowl of the Central Flyway, providing habitat for approximately two to three million ducks and over a million geese (DU, 2008). In this region, migratory waterfowl intermingle with wild resident species, such as the mottled duck (*Anas fulvigula*), and are in close contact with poultry operations and humans, primarily hunters (Bellrose, 1978; Miller, 2007). Until now, few studies involved waterfowl on their wintering grounds or nonmigratory waterfowl during the winter, particularly along the Gulf Coast, and most published studies were limited to a few species [teals (*Anas crecca, A. cyanoptera, A. discors*),

gadwall (*A. strepera*), mottled duck, northern pintail (*A. acuta*), and mallard (*A. platyrhynchos*)] and were limited in time, sometimes covering only a single season of one year (Hanson et al., 2005; Stallknecht et al., 1991; Stallknecht et al., 1990) . This project provides information regarding AIVs in nature in an understudied region, the wintering grounds of the Central Flyway, the Texas mid-Gulf Coast throughout four consecutive hunting seasons.

We collected 6,823 swabs over four years (2005–2006: 1,460; 2006–2007: 2,171; 2007–2008: 2,424; and 2008–2009: 768) from 30 different potential host species. Most samples (88.3%) were from dabbling ducks (genus *Anas*), while diving ducks (genus *Aythya*) accounted for 5.0%, and geese (genera *Anser, Chen* and *Branta*) 3.0% of the samples tested, with waterfowl (*Anatidae*) comprising 98.7% of samples. Of the waterfowl (*Anatidae*) sampled, 1.8% of the samples were from non-migratory dabbling ducks (genus *Anas*). The remaining samples were collected from six other potential host species, and in three cases only one sample was tested per species.

Over the four-year course of our study, we performed virus isolation on 5,013 samples and rRT-PCR on 6,823 samples resulting in 146 AIV isolates, of which five were obtained from rRT-PCR negative samples. Similar to most surveillance studies, we found no significant difference in AIV infection based on host sex, but did find that juveniles were more likely to be positive for AIV than adults. We also documented that dabbling ducks were more likely to be positive for AIV than diving ducks, although not all dabbling ducks are equally likely to be positive. In many studies, mallards have become a primary species of interest not only because of their susceptibility to H5 and

H7 subtypes, but also because of their abundance and relative ease of capture (Bellrose, 1978; Dusek et al., 2009; Jourdain et al., 2010; Munster et al., 2007; Olsen et al., 2006; Wallensten et al., 2007). Our data and other researchers' reports indicate that blue-winged teal, green-winged teal, or northern shoveler are better species of choice for AIV surveys in the Texas mid-Gulf Coast and California (Ferro et al., 2008; Hanson et al., 2005; Siembieda et al., 2010; Stallknecht et al., 1990) due to their greater abundance and relatively high AIV prevalence in these regions.

We observed significant differences in prevalence estimates based on rRT-PCR compared to virus isolation. Differences observed in apparent prevalence estimates over the four years of our study between virus isolation (0.5, 1.3, 3.9, and 0.7%) and rRT-PCR (5.9, 6.5, 11.2, and 5.5%) are not surprising because rRT-PCR can detect genome fragments and does not require the presence of intact infectious particles as does viral isolation. We determined that screening samples by rRT-PCR and performing virus isolation only on the rRT-PCR-positive samples resulted in prevalence estimates that were nearly identical to those derived from parallel testing. Thus our data support the commonly employed practice of using rRT-PCR as a screening tool and performing virus isolation only on rRT-PCR positive samples (Cattoli et al., 2007; Dusek et al., 2009; Munster et al., 2009; Zohari et al., 2008).

Unlike previous reports of seasonal variation in AIV prevalence (Halvorson et al., 1985; Munster et al., 2009; Stallknecht et al., 1990), we documented differences in prevalence estimates among months using rRT-PCR only during 2008–2009 and by virus isolation only during 2006–2007 and 2007–2008. Additional surveys including all

months of the year as well as other wintering grounds and year-round sampling of resident species, such as the mottled duck, would provide valuable data regarding AIV prevalence and persistence in these understudied areas.

Several of the AIV subtypes we identified are common in North America. The H3, H4, and H6 subtypes are considered the most common in North America, while H1, H2, H7, H10, and H11 are less common; H3N8 (22.8%), H6N4 (20.8%), and H4N6 (12.5%) were the most common subtype combinations isolated from ducks (Krauss et al., 2004). In our study, 24 of the 39 (61.5%) H4 isolates and 17.0% of all isolates were H4N6; interestingly, this subtype combination had previously been reported in Louisiana, but not in Texas (Hanson et al., 2005; Stallknecht et al., 1990). The H3N8 subtype combination comprised 80.0% of the H3 subtypes identified and 11.3% of all viruses isolated. Another frequently identified subtype combination was H10N7, which accounted for 61.9% of all H10 viruses isolated and 9.2% of all isolates. Only one H6N4 subtype combination was isolated throughout the four years of this study. The absence of certain subtypes or subtype combinations may be due to the virus not circulating in a population or our inability to detect it. Further, all surveillance studies sample a subset of a population rather than testing every individual, so samples typically cannot be collected probabilistically for logistical reasons when working with wild animals, particularly migratory water birds. For these reasons, one must be cautious about extrapolating the results of such studies to the populations of waterfowl sampled.

The viruses sequenced as part of this study were all low pathogenic AIVs isolated from presumably healthy migratory waterfowl, primarily dabbling ducks.

Molecular sequence analysis supported the identification of LPAIV in that no insertions of multiple basic amino acids at the cleavage site were identified. All molecular sequencing returned results similar to the classical subtyping methods of hemagglutinination inhibition (HI) and neuraminidase inhibition (NI) assays with the exception of one NA gene. This isolate that was identified as an N2 using classical NI assays was identified as an N1 by molecular sequencing. Most isolates sequenced herein grouped relatively closely together thus demonstrating sequence similarity. This is not surprising in that many of the isolates were collected within the same hunting season and within the same wildlife management area.

The sequences of our isolates following phylogenetic analysis showed a separation from those of Eurasian origin with all subtypes examined except H6. Previous studies indicated replacement of the North American clade H6 with the Eurasian clade H6 occurred in North America, particularly in the Pacific Flyway (zu Dohna et al., 2009). Our results indicate the same is true in the Central Flyway, in that they are most closely related to H6 isolates from the Pacific Flyway and group with Eurasian H6 lineages. Given the close proximity and the slight overlapping of the Central and Pacific Flyways, the similarity in H6 sequences is not entirely surprising. Other studies have shown H6 to have a wider host range than other subtypes which could enable this subtype to spread between and within hemispheres (Munster et al., 2007). Most surprising is this phenomenon has not been reported with other gene segments or HA subtypes, given the overlap of migratory flyways worldwide. Perhaps as more isolates

are identified and full genome sequencing becomes more commonplace more intercontinental exchange of AIV genes will be discovered.

A problem with genetic analyses thus far, particularly with AIVs, is that these analyses rely on consensus sequences, which means the nucleotide shown is the most common among all the genomes within a host (Holmes and Grenfell, 2009). Additionally, when analyzing viral isolates, selection already has occurred for the virus that grows best in the isolation system (embryonated chicken eggs in the case of AIV), thereby eliminating sequence information that may be critical to understanding the evolution of influenza viruses under study. Additionally, by sequencing from isolates, we miss all information that could be gained from those viruses that occurred in sampled individuals but were not isolated, such as the rRT-PCR positive samples that are virus isolation negative. Perhaps as newer technologies and techniques are developed, these issues will be resolved.

Many gaps in knowledge regarding AIVs still exist, despite the >60 years that have passed since AIV was first characterized. With the recent emergence of large-scale sequence analyses, high-throughput sequencing of AIV isolates now is possible and may provide valuable information about how influenza viruses persist and change in nature . Further studies involving molecular characterization and comparison of the same influenza virus subtype from different regions along a flyway also should provide important information regarding the evolution of AIVs in nature. Similarly, studies following target species (those identified as having a high prevalence), throughout their migration, could provide valuable information regarding persistence of AIV in these

species. Finally, studies covering multiple consecutive years at the same area, such as on waterfowl wintering grounds, will enable better understanding of the ecology and evolution of influenza viruses and thus how these viruses persist in nature over time, particularly over winter.

In sum, this is the first multiyear study of avian influenza viruses on waterfowl wintering grounds of the Central Flyway. Our data provides temporal information on AIV prevalence and subtype diversity for a historically understudied area of North America. Additionally, the sequences we obtained will be deposited into publicly accessible databases and available to all for further analyses. This study contributes to knowledge of influenza virus prevalence on waterfowl wintering grounds in Texas and provides information that will contribute to the elucidation of subtype prevalence, evolution, and persistence of AI in wild waterfowl, including migratory and non-migratory species. This study provides a foundation for future work; there is much that remains to be discovered.

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VITA

Name: Pamela Joyce Bloomer Ferro

Education: Ph.D., Veterinary Microbiology, Texas A&M University, 2010
M.S., Veterinary Microbiology, Texas A&M University, 2001
B.S., Sociology, Texas A&M University, 1995

Address: Department of Veterinary Pathobiology
C/O Dr. Blanca Lupiani
Texas A&M University
College Station, TX 77843-4467

Email Address: pj.ferro87@gmail.com